Rab GDP-dissociation inhibitor gdiA is an essential gene required for cell wall chitin deposition in Aspergillus niger

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ABSTRACT
The cell wall is a distinctive feature of filamentous fungi, providing them with structural integrity and protection from both biotic and abiotic factors. Unlike plant cell walls, fungi rely on structurally strong hydrophobic chitin core for mechanical strength together with alpha- and beta-glucans, galactomannans and glycoproteins. Cell wall stress conditions are known to alter the cell wall through the signaling cascade of the cell wall integrity (CWI) pathway and can result in increased cell wall chitin deposition. A previously isolated set of Aspergillus niger cell wall mutants was screened for increased cell wall chitin deposition. UV-mutant RD15.8#16 was found to contain approximately 60% more cell wall chitin than the wild type. In addition to the chitin phenotype, RD15.8#16 exhibits a compact colony morphology and increased sensitivity towards SDS. RD15.8#16 was subjected to classical genetic approach for identification of the underlying causative mutation, using co-segregation analysis and SNP genotyping. Genome sequencing of RD15.8#16 revealed eight SNPs in open reading frames (ORF) which were individually checked for co-segregation with the associated phenotypes, and showed the potential relevance of two genes located on chromosome IV. In situ re-creation of these ORF-located SNPs in a wild type background, using CRISPR/Cas9 genome editing, showed the importance Rab GTPase dissociation inhibitor A (gdiA) for the phenotypes of RD15.8#16. An alteration in the 5′ donor splice site of gdiA reduced pre-mRNA splicing efficiency, causing aberrant cell wall assembly and increased chitin levels, whereas gene disruption attempts showed that a full gene deletion of gdiA is lethal.

1. Introduction
The fungal cell wall is an essential organelle that sets apart filamentous fungi from other eukaryotes. Mainly comprised of α-1,3-glucans, β-1,3-glucans, β-1,6-glucans and mixed β-1,3/1,4 varieties, chitin (β-1,4-linked-N-acetyl glucosamine), galactomannan and glycoproteins, the cell wall is continuously being built, remodeled, broken down and re-built to accommodate various stages and challenges of the filamentous lifestyle. For extensive review on cell wall organization and biosynthesis we refer to both Free (2013) and Gow et al. (2017), with the references therein. In addition to the majority of glucans, chitin plays a structurally important part of the fungal cell wall for mechanical strength. At the plasma membrane, chitin synthases assemble the UDP-N-acetyl-glucosamine monomers into chitin polymers by extrusion of nascent chitin chains into the periplasmic space. Careful coordination of sufficient precursor and available chitin synthases at the plasma membrane, determine both the rate of which chitin is deposited into the cell wall and chitin chain length (Kang et al., 1984; Keller and Cabib, 1971; Orlean and Funai, 2019; Peter, 1987; Sburlati and Cabib, 1986). Seven clearly defined classes of chitin synthases (CHSI-CHSVII) are known to exist in filamentous fungi, some of which have been studied in detail, and have been shown to be correlated with morphogenesis and adaptation to ecological niches (Liu et al., 2017). Encompassing many different classes of chitin synthases, fungi temporally and spatially regulate expression during different stages of development. Additional post-translational activation of certain chitin synthases, involving glycosylation (Santos and Snyder, 1997; Trilla et al., 1999), phosphorylation (Valdivia and Schekman, 2003) and proteolytic cleavage of zymogenic chitin synthases (Choi et al., 1994) all contribute to tight regulation of chitin deposition in the cell wall.

As a result of this complex organization, levels of cell wall chitin are generally well maintained, and fluctuations in chitin content mainly differ depending differing stages of life cycle, environmental cues, mycelial age, available nutrients and cultivation conditions, hypoxia...
and stress (Lord and Vyas, 2019; Pochanavich and Suntornsr, 2002). Cell wall stress often results in increased cell wall chitin content through activation of the cell wall integrity (CWI) signal transduction pathway (Fortwendel et al., 2010; Heilmann et al., 2013; Ram et al., 2004; Walker et al., 2015, 2008). This conserved, natural response in filamentous fungi often involves a compensatory increase in both cell wall chitin and alpha-glucan deposition. Specifically, under cell wall stress conditions in A. niger it was reported that levels of both agsA (alpha-glucan synthase A) (Damveld et al., 2005) and gfaA (glutamin-fructose-6-phosphate-amidotransferase A) (Ram et al., 2004) are induced. The expression of the former was previously used in a cell wall stress reporter system to identify mutants with a constitutively activated cell wall stress response in a UV-screen (Damveld et al., 2008). We identified mutants from this cell wall mutant library that display an increased cell wall chitin content (van Leeuwe et al., manuscript in revision). Resultant from this screen, mutant RD15.8#16 was identified as a strain with increased cell wall chitin levels.

In the quest to identify the genotype related to the cell wall phenotype of RD15.8#16, we took a classical genetics approach combined with genome sequencing. A lacking established sexual cycle of A. niger prevents traditional type crossings, however, a parasexual cross was used instead to obtain segregants (Arentshorst and Ram, 2018; Pontecorvo et al., 1953; Swart et al., 2001). It is important to note in this approach that, unlike a conventional sexual cycle, cross-over events are singularly mitotic and are therefore relatively rare. When cross-over events are absent, co-segregation analysis is only indicative of the linked chromosome, rather than a specific gene or genomic region.

In this study, we performed co-segregation analysis of the RD15.8#16 phenotype by selecting segregants that displayed either wild type or the RD15.8#16 phenotypes. Segregants with either of the two phenotypes were checked for presence of SNPs that are unique to RD15.8#16. Comparative SNP analysis revealed the chromosomal distribution among segregants and identified the exclusive co-segregation of markers on chromosome IV with the RD15.8#16 phenotype. With the use of a previously reported CRISPR/Cas9 gene editing system (van Leeuwe et al., 2019), we performed in situ SNP editing of the endogenous wild type allele to re-create the respective mutant alleles on chromosome IV. Re-creation of mutant alleles in a wild type genetic background revealed that inefficient and/or aberrant splicing of Rab GDP-dissociation inhibitor (gdiA) is responsible for the phenotype of RD15.8#16.

2. Materials and methods

2.1. Strains, media, growth conditions

Strains used in this study can be found in Table 1. All media were prepared as described by Arentshorst et al. (2012). In all cases, minimal medium (MM) containing 1% (w/v) glucose, 1.5% agar and was supplemented when required with 10 mM uridine and 2.5 µg/mL nicotinamide. To test the presence or absence of the amdS gene, MM acetamide agar (MM-AA) was used as described by Arentshorst et al. (2012). Complete medium (CM) contained 1% (w/v) glucose, 1.5% agar (Scharlau, Barcelona, Spain), 0.1% (w/v) casamino acids and 0.5% (w/v) yeast extract in addition to MM. Strains were inoculated from −80 °C glycerol stocks onto fresh CM plates and were allowed to grow and sporulate for 5–7 days at 30 °C, prior to spore harvesting. Spores were harvested by addition of 15 mL of 0.9% (w/v) NaCl to CM spore plates and were carefully scraped from the surface with a cotton swab. Spore solutions were poured over sterile cotton filters (Amplitude™ Ecocloth™ Wipes, Contec Inc., Spartanburg, SC, USA) to remove large mycelial debris. Spore solutions were counted using Bio-Rad TC20™ Automated Cell Counter (Bio-Rad Laboratories, Inc. USA) using Counting Slides, Dual Chamber for Cell Counter (Cat#145-0011, Bio-Rad Laboratories, Inc. USA).

2.2. SDS sensitivity assays

Wild type, mutants and segregants were tested on different concentrations of SDS. Using a 10% (w/v) SDS stock 0.004%, 0.0045% and 0.005% SDS was added to MM supplemented with uridine and nicotinamide. Spore stocks were created as described above. Spores were counted, serially diluted into 2000, 200, 20 and 2 spores/µL and 5 µL of respective dilutions were spotted on MM SDS plates. The plates were incubated for 96 h at 30 °C prior to scoring phenotypes.

SDS sensitivity was tested with un-normalized spore concentrations during the segregants screen. A total of 200 segregants and controls were streaked twice on MM + U + N and spores were harvested from a single colony using a pre-wetted (0.9% NaCl) cotton swab. Cotton swab containing spores was dipped and swirled in 0.5 mL 0.9% NaCl to dissolve spores. For each segregant and control strain, 5 µL spore solution was spotted on plates for testing phenotypes (see Section 2.4).

2.3. Cell wall isolation and chitin analysis

2.3.1. Cell wall isolation

Strains were cultured to obtain equally aged spores as described above. To 25 mL of liquid CM (100 mL Erlenmeyer flask), a final concentration of 10^6 spores/mL was added and grown overnight for 17 h at 30 °C, 200 rpm. Mycelium was harvested by applying a vacuum over a Whatman™ Glass Microfiber Filter (GF/C™) (diameter 47 mm, CAT No.1822-047, Buckinghamshire, UK) to remove medium and capture and dry the mycelium. Dried mycelium was frozen in liquid N2 prior to grinding in order to break open the cells with mortar and pestle into a fine powder. Next, samples were washed to remove intracellular debris and proteins: washing occurred by addition of 50 mL 1 M NaCl, followed by three washing steps with 50 mL MQ. In both cases washing involves vigorous shaking and vortexing with 25 mL volume in 50 mL plastic tubes (114 × 28 mm, Sarstedt AG & Co. KG, 62.547.254). Subsequently, the remaining 25 mL volume was added followed by repeated shaking and vortexing. Cell wall suspensions were centrifuged at 3500 rpm for 10 min. to pellet cell walls. Supernatant was carefully discarded prior to the next washing step. Cell walls were lyophilized after washing steps for 48 h.

2.3.2. Cell wall hydrolysis and chitin analysis

Chitin was measured as total glucosamine and was performed based on the principle of the Morgan-Elson protocol (Elson and Morgan, 1934) and was adapted for higher-through-put analysis, using a 96-well plate reader. See appendix (Supplementary document 1) for a detailed description of the protocol. Due to variability between separate experiments in absolute glucosamine content, a wild type control was always included every time to compare relative differences. Cell wall glucosamine measurements from independent replicate experiments are expressed as means ± SEM. The statistical analysis was carried out using software R studio (Version 1.1.456) (RStudio: Integrated Development for R. RStudio, Inc., Boston, 2016). For total cell wall glucosamine experiments, we used one-way ANOVA. Significant differences between groups were subjected to posthoc Tukey multiple-comparisons analysis. Significance levels are indicated as p < 0.05 (*), p < 0.005 (**), p < 0.001 (***), and p < 0.0001 (****).

2.4. Parasexual cycle and segregant analysis

Formation of heterokaryons and selection for diploids was performed as described previously described (Arentshorst and Ram, 2018). Requirements for this procedure are for each strain to have separate auxotrophic deficiencies and different color markers from one of the three known complementation groups involved in melanin production: fawn (hwa, NRRL3_00462, An09g05730), olive (olvA, NRRL3_01039, An14g05350) or brown (brmA, NRRL3_0140, An14g05370) colored (Jorgensen et al., 2011). As such, two haploid strains are coerced to fuse
without supplementation for their respective auxotrophic deficiencies. This process yields a heterokaryotic, prototrophic mycelium in which karyogamy can occur at a very low frequency, resulting in a diploid strain. Due to the primarily uninuclear nature of asexual spores, color markers help identify whether nuclei have fused, and become black as a result of complementing alleles from the other chromosome, becoming asexual spores, and taken up into 500 µL 0.9% (w/v) NaCl. For each segregant, 5 µL spore solution was spotted on MM + uridine and MM-AA + uridine + nicotinamide and on aseptically to the expected size of approximately 500–700 bp. Clustering and DNA sequencing using the Illumina cBot and HiSeq 4000 was performed according to manufacturer’s protocols. A concentration of 3.0 nM of DNA was used. HiSeq control software HCS v3.4.0 was used. Image analysis, base calling, and quality check was performed with the Illumina data analysis pipeline RTA v2.7.7 and Bcl2fastq v2.20. SNP calling was performed according to GenomeScan Guidelines Small Variant Analysis v3.0. The Variant Call Format (VCF) files were manually analyzed by the authors. Frequency score of identical SNP call boundary was set to ≥ 0.75, while sequencing depth was left unsel ected.

### 2.6. Co-segregation analysis of SNPs

Approximately 400–500 bp long PCR products surrounding the SNP in question were amplified with primers listed in Primer Table. An additional nested primer was designed for each PCR product used for sequencing. Sequencing of individual SNPs was performed using Sanger sequencing (Macrogen Europe, Amsterdam, The Netherlands). Analysis of sequencing data and alignments were performed in Benchling [Biological Software] 2019.

### 2.7. Single gene knockouts using split marker fragments

MA169.4 (Table 1) was transformed after protoplastlation as described previously (Arentshorst et al., 2012). Using the split marker approach for single gene knockouts, entire ORFs were deleted by replacement with the Aspergillus oryzae pyrG (AOpyrG) selection marker (Arentshorst et al., 2015). Flanks were generated via PCR using N402 genomic DNA as template and primers as described in Primer Table. AOpyrG fragments were obtained from plasmid pAO4-13 (de Ruiter-Jacobs et al., 1989) with primers as described in Primer Table. Fusion PCR was used to generate split marker fragments containing AOpyrG. Approximately 2 µg of DNA per flank was added to protoplasts for transformation. Transformation plates were incubated on MMS for 6 days at 30 °C. Transformed colonies were single streaked on MM twice for purification and were genotyped using diagnostic PCR (data not shown).
2.8. SNP re-creation in a wild type background using CRISPR/Cas9 gene editing

SNPs were introduced in a wild type (MA234.1) background using CRISPR/Cas9 mediated gene editing with a marker-free repair DNA fragment (van Leeuwe et al., 2019). All primers are listed in Primer Table. Primers OTL479 and OTL480 were used in combination with pTE1_rev and pTE1_for, respectively, to obtain a sgRNA construct to target the wild allele NRR3_05482 in MA234.1. Similarly, for NRR3_06010, primers OTL477 and OTL476 were used in combination with pTE1_rev and pTE1_for, respectively, to obtain a sgRNA construct to target the wild allele NRR3_06010 in MA234.1. Plasmids pTLL108.1 and pTLL109.2 were used as template DNA for sgRNA flanks. Flanks were fused through PCR to obtain sgRNAs, and cloning of the sgRNAs into pFC332 resulted in pFC332_NRR3_05482-sgRNA and pFC332-NRR3_06010-sgRNA. Marker-free repair DNA fragment for NRR3_05482 was obtained through fusion PCR, 5’ flank was amplified using OTL481 and OTL482, whereas 3’ flank was amplified with OTL483 and OTL484. OTL482and OTL483 contained a single mismatch to introduce a point mutation (see Section 3.5) and allowed sufficient overhang for generation of a fusion construct. Marker-free DNA fragment repair for NRR3_06010 was amplified from RD15.8 as template DNA, using OTL485 and OTL486. CRISPR/Cas9 plasmid transformations were performed after protoplastation as described previously (van Leeuwe et al., 2019), using a pFC332 (hph) plasmid (Nødvig et al., 2015): 2 µg of Cas9-sgRNA plasmid with approximately 2 µg of repair DNA fragment for transformation. Transformation plates were incubated on MMS with 200 µg/mL hygromycin for 7 days at 30 °C. Transformed colonies were single streaked on selectable MM with 100 µg/mL hygromycin to select for the presence of the Cas9-sgRNA plasmid. Next, a single colony was picked and transferred to non-selective MM medium to allow loss of the Cas9-sgRNA plasmid. A third streak of a single colony on both MM and MM with 100 µg/mL hygromycin uridine was performed as a control for loss of plasmid. DNA from plasmid-cured strains was isolated as described by Arentshorst et al. (2012), using mortar and pestle to grind the mycelium in liquid nitrogen. Genotypes were confirmed using diagnostic PCR. Diagnostic PCR fragments from wild type (RD15.8), mutant (RD15.8#16) and transformants were sent for sequencing to check for SNP alterations (Macrogen Europe, Amsterdam, The Netherlands).

2.9. RNA isolation and RT-PCR for cDNA

RNA was isolated and column purified according to Park et al. (2016). Complementary DNA (cDNA) was obtained using the Quantitect Reverse Transcription Kit (Qiagen). In short, 1 µg of RNA was added to 2 µL of gDNA Wipeout Buffer and RNase-free water was added to a total volume of 14 µL. After incubation for 2 min at 42 °C, the reactions were placed on ice. Then Reverse-transcription master mix (1 µL), Quantscript RT Buffer (4 µL) and RT Primer Mix (1 µL) were added and the RT-reaction was performed for 15 min at 42 °C, followed by a heat inactivation step of 3 min at 95 °C. 1 µL of the resulting cDNA library was used as template in subsequent PCR experiments.

3. Results

3.1. RD15.8#16 displays a compact colony morphology, SDS sensitivity and increased cell wall chitin

RD15.8#16 was selected from a previously obtained set of cell wall stress mutants (Damveld et al., 2008), and was found in a screen for strains with an increased cell wall chitin content (van Leeuwe et al., 2019, manuscript submitted). Increased cell wall chitin was suggested by increased Calcofluor White (CFW) staining (Fig. 1A). Next, total glucosamine content from cell wall dry weight was measured for the wild type strain N402 (159 µg/mg ± 5.01, n = 3), parental strain RD15.8 (163 µg/mg ± 8.73, n = 3) and UV-mutant RD15.8#16 (263 µg/mg ± 7.80, n = 3) and is depicted in Fig. 1B. Whereas the wild type and parental reporter strain have equal amount of cell wall glucosamine, RD15.8#16 shows a respective increase of 61% chitin. In addition to the cell wall phenotype, the mutant has a compact colony morphology and has increased sensitivity towards SDS (Fig. 1C).

3.2. A parasexual cross and segregation analysis to isolate linkage to SDS sensitivity

To perform a parasexual cross and subsequent detection of segregation of diploid strains, we used a derivative strain of RD15.8#16 harboring ΔbrnA, pyrG, strain TLF54 (see section 2.4). A previously obtained olive-colored, nicB- wild type strain JN6.2 (ΔnicB::AOpyrG, ΔolvA::hgyB, (Niu et al., 2016), Table 1) was used to perform a parasexual cross with TLF54.

Fig. 1C shows an SDS sensitivity phenotype for RD15.8#16 used for segregants screening. Prior to segregant analysis, both parental strains JN6.2 and TLF54 were checked against their non-auxotrophic, non-color deficient counterparts RD15.8 and RD15.8#16, respectively. As is evident from Fig. 2, both parental strains for the parasexual cross show the same level of SDS sensitivity as their parental counterparts. Additionally, colony morphologies of RD15.8#16 and TLF54 are identical and show a more compact growth style than both JN6.2 and RD15.8.

Morphologies of wild type JN6.2, TLF54, original mutant RD15.8#16 and diploid TLF92 are shown on CM and CM + 0.5 µg/mL benomyl in Supplementary Fig. 1. The diploid strain TLF92 forms black spores, indicating that all spores contain two sets of chromosomes by color complementation of brnA and olvA. On CM + 0.5 µg/mL benomyl, the diploid strain forms sectors of both brown and olive colors that represent chromosomal loss from diploid to haploid (Supplementary Fig. 1).

In total 200 segregants were screened for SDS sensitivity by spotting spores on MM + U + N + 0.005% SDS, and for colony morphology resembling TLF54. Only two out of 200 segregants were found to display the same compact phenotype of TLF54 and were prone to SDS sensitivity (Supplementary Fig. 2A and B, segregants ΔbrnA#53 and ΔbrnA#90). Two randomly picked segregants, ΔolvA#27 and ΔolvA#41 resembling both wild type-like morphology and wild type-like SDS sensitivity, were taken in addition to ΔbrnA#53 and ΔbrnA#90 for a closer comparative analysis SDS sensitivity with normalized spore concentrations (Fig. 3A). Evidently, we confirmed that ΔbrnA#53 and ΔbrnA#90 are indeed equally sensitive to SDS as TLF54. The chitin content of the cell wall was analyzed by determining the total cell wall glucosamine content of ΔbrnA#53 and ΔbrnA#90, parental strains JN6.2, TLF54 and segregants ΔolvA#27, ΔolvA#41. Fig. 3B shows a comparative analysis of glucosamine content of the aforementioned strains versus the wild type parent JN6.2. Both ΔbrnA#53 and ΔbrnA#90 showed a significant increase in cell wall glucosamine versus JN6.2, but were not significantly different from TLF54. In addition, segregants ΔolvA#27 and ΔolvA#41 do not differ significantly from JN6.2. These data suggest that both ΔbrnA#53 and ΔbrnA#90 inherited all the associated phenotypes of TLF54 and that the SDS sensitivity and increased cell wall chitin are caused by the same mutation.

Due to very unequal segregation of mutant traits, we checked for chromosome distribution among segregants based on the other available genetic markers. As brnA and olvA are located adjacent to each other on chromosome I, we initially picked a total of 100 brown and 100 olive segregants segregating TLF92 diploid strain to obtain equal numbers of segregants in which one of the copies of chromosome I was present. Equal distribution of wild type and mutant of chromosomes was also checked for chromosome III and VII using the markers located on either chromosome III (ΔkuA::amds: JN6.2) or chromosome VII (nicB, TLF54), by scoring all 200 segregants for presence or absence of nicB+ and amds+. Segregation of nicB+/− was found to be 102/98, whereas amds+/− segregation was scored to be 84/116
These data suggested that the segregation of TLF92 occurred at an approximate 50/50 ratio of chromosomal co-segregation for the genetically marked chromosomes.

### 3.3. Genome sequencing reveals eight ORF-located SNPs in RD15.8#16

To identify the genotypic relation of the cell wall phenotype of RD15.8#16, we performed genome sequencing of TLF54; the derivative of RD15.8#16 used as mutant parent in the parasexual cross and compared it with the genome sequence of RD15.8. SNP calling was performed as described in Section 2.5; a total of 44 SNPs and 9 indels were identified across the genome of TLF54 (Supplementary Table 2). Eight SNPs and two insertions were identified to be inside ORFs and nucleotide changes and their respective effect on protein sequence are listed in Table 2. In addition to coding sequence changes, two genes were found to harbor intron-located mutations.

### 3.4. Co-segregation analysis of SNPs reveals importance of chromosome IV

Due to the low number of segregants with the mutant phenotype of (Supplementary Table 1). These data suggested that the segregation of TLF92 occurred at an approximate 50/50 ratio of chromosomal co-segregation for the genetically marked chromosomes.

![Fig. 1. Chitin content and morphology of UV-mutant and wild types. (A) Chitin content of N402, RD15.8 (parent) and RD15.8#16 are shown (n = 3). (B) Growth morphology on MM, and SDS sensitivity on MM + SDS for RD15.8 and RD15.8#16. Statistical methods and significance is described in section 2.3.2. Listed significant differences are compared to N402. Abbreviation n.s. refers to not significant.

![Fig. 2. Morphology of strains involved in parasexual cross. SDS sensitivity spot assay of RD15.8, JN6.2, RD15.8#16 and TLF54 on 0.004% and 0.005% SDS in Minimal Medium (MM) with 10 mM uridine (U) and 2.5 µg/ml Nicotinamide (N). From left to right, spore count equals 10^4, 10^3, 10^2 and 10^1. Strains were incubated 96 h at 30 °C.](image)
TLF54, we were unable to perform bulk segregant analysis (BSA) and decided to identify relevant SNPs by performing a SNP co-segregation analysis. Initially, SNPs were determined by sequencing the ORF-located SNPs (Table 2, with the exception of pyrG) in mutant segregants ΔBRNA#53 and ΔBRNA#90, and two segregants that did not display the mutant phenotype (ΔvolvA#27 and ΔvolvA#41).

Sequenced SNPs of parental strains and segregants are displayed schematically in Fig. 4. Yellow bars represent the SNPs of the wild type, whereas green bars indicate the SNP identified for TLF54. The co-segregation analysis indicated that SNPs associated with chromosome I and IV co-segregate with the mutant phenotype (Fig. 4). However, note that the SNP on chromosome I (NRRL3_01084) is located on the same chromosome arm as either color marker ΔvolvA (wild type SNP) or ΔbrnA (mutant SNP), making co-segregation of the ΔbrnA color marker, and not the cell wall mutant phenotype, a possible consequence of genetic linkage.

To check whether mutant SNPs on chromosome I (NRRL3_01084) or on chromosome IV (NRRL3_05482 and NRRL3_06010) are co-segregating with the phenotypes, a second set of segregants with wild type-like phenotypes was included for SNP sequencing shown in Fig. 4. Only wild type SNPs were found for both NRRL3_05482 and NRRL3_06010 on chromosome IV in segregants that do not display the TLF54 phenotype. These data suggest that the SNPs found on chromosome IV are associated with the phenotypes of RD15.8#16.

3.5. SNPs re-creation shows that the mutant allele of NRRL3_06010/gdiA is responsible for the phenotype of RD15.8#16

The SNP co-segregation analysis showed the SNPs on chromosome IV to be associated with either the wild type or mutant phenotype. In addition to NRRL3_05482 and NRRL3_06010, TLF54 harbors three additional SNPs on chromosome IV (Supplementary Table 2), but were all found to be outside of promoter or coding regions. Therefore, we considered the two genes NRRL3_05482 and NRRL3_06010 on chromosome IV in segregants that do not display the TLF54 phenotype. These data suggest that the SNPs found on chromosome IV are associated with the phenotypes of RD15.8#16.

Using a split marker approach as described in Section 2.7, we attempted to create full gene knockouts. Transformants of both ΔNRRL3_05482::ΔOpyrG and ΔNRRL3_06010::ΔOpyrG in MA169.4 showed low levels of sporulation and single streaking on either selective or non-selective medium often did not show colony forming units derived from single spores, suggesting these genes are essential for growth and the null allele only allows growth through heterokaryotic rescue (Osmani et al., 2006). Transformants that did grow after re-streaking were expected to contain ectopically integrated selection markers, as diagnostic PCR did not show correct deletion of ORF (data not shown).

Based on these results we opted to re-create the mutant SNPs in a wild type background using CRISPR/Cas9 genome editing. This was achieved by targeting either NRRL3_05482 or NRRL3_06010 in the wild type MA234.1 (Table 1) with respective plasmids pFC332_NRRL3_05482-sgRNA and pFC332_NRRL3_06010-sgRNA, and simultaneously present a linear repair DNA fragment from TLF54 that contains the SNPs in question, for homologous recombination.

For NRRL3_06010, we exploited the location of the +2 intronic SNP to create a sgRNA target that targets the wild type SNP, but does not recognize the mutant SNP located on the DNA repair fragment. The +2 intronic G/GT–> G/GG mutation of the NRRL3_06010 mutant allele is situated at the second nucleotide upstream from the PAM site of the NRRL3_06010-sgRNA target sequence. A schematic representation of this approach for NRRL3_06010 is shown in Supplementary Fig. 3A and 3B. MA234.1 was transformed with pFC332_NRRL3_06010-sgRNA and a 2142 bp linear DNA repair fragment (amplified from genomic DNA of TLF54, with primers OTL485 and OTL486 (Primer Table). Sequencing of the NRRL3_06010 locus in transformants revealed the successful integration of the linear repair DNA fragment (data not shown), yielding strain AG3 with the NRRL3_06010 mutant allele SNPs at the +2 and +7 intronic position (Table 1).

The SNP located in NRRL3_05482 mutant allele did not provide a favorable target site for sgRNA design. Instead, a target that lies 317 bp downstream was used to create plasmid pFC332_NRRL3_05482-sgRNA. To omit recognition of the NRRL3_05482-sgRNA-Cas9 ribonucleoprotein (RNP) complex of the linear DNA repair fragment obtained from NRRL3_05482 mutant allele, we introduced a silent mutation (ACG to ACA: NRRL3_05482 Thr318(ACA)) at the target site of AGC (–> ACA, Thr) in the target site at the 10th nucleotide upstream from the PAM site (5′– TGG – 3′). This was achieved by in situ single mismatch PCR, creating two flanks that were subsequently fused by fusion PCR. A schematic overview of this approach is displayed in Supplementary Fig. 3C. Using the genomic DNA of TLF54, we used primers to introduce a G to A transition using primers listed in Primer Table. The combined flanks yield a 2499 bp linear DNA repair fragment from NRRL3_05482 mutant allele with an additional SNP in Thr318 (ACG to ACA: NRRL3_05482 Thr318(ACA)) at the target site of NRRL3_05482-sgRNA, and was sequenced to confirm this addition (data not shown). MA234.1 was transformed with pFC332_NRRL3_05482-sgRNA and repair DNA fragment NRRL3_05482Thr318(ACA). Transformants were confirmed using sequencing (data not shown), yielding strain AG1.

Equally aged sporue stocks of strains RD15.8, RD15.8#16, AG1 and AG3 were harvested, counted and diluted for a SDS sensitivity spot
assay. On MM, it is evident that RD15.8 and AG1 show a similar growth phenotype, whereas AG3 displays the same compact colony phenotype as RD15.8#16 (Fig. 5). Moreover, AG3, harboring the mutant allele of NRR3L.06010, also shows sensitivity towards SDS as seen for RD15.8#16 across a range of SDS concentrations, whereas AG1 showed wild type like sensitivity. In addition, measured glucosamine content from isolated cell walls were found to be identically increased for both RD15.8#16 and AG3 over RD15.8 (data not shown). Interestingly, NRR3L.06010 encodes a homologue of the previously identified Aspergillus nidulans gdiA (ANS959), a Rab GDI dissociation inhibitor (Abenza et al., 2010; MacKenzie et al., 2005).

3.6. Analysis of gdiA mRNA from RD15.8#16 reveals both inefficient and alternative splicing

SNPs in the gdiA mutant allele of RD15.8#16 are intronic, showing two SNPs near the 5’ donor splice site of intron 2. To assess effects of these mutations on splicing of gdiA pre-mRNA (SNPs at the +2 and +7 position, G/GTAGGGA to G/GGAGGGG of the second intron) in RD15.8#16, RNA was isolated from shake flask cultures of both RD15.8 and RD15.8#16 and cdna was generated using RT-PCR as described in Section 2.9. The cdNA of gdiA was amplified using PCR (Primer Table) and revealed PCR products for both RD15.8 and RD15.8#16 (data not shown). Next, the purified PCR products were cloned into a pJET1.2/blunt vector. Fourteen clones of RD15.8#16 and of two clones of RD15.8 gdiA mRNA (as control) were sequenced using primer6010_P12r (Primer Table). The two cDNA clones from RD15.8 both carried the fully processed cDNA (https://gb.fungalgenomics.ca/portal/view/geneModelView.php?fullName=NRRL3_06010). Visualization of splicing in wild type and mutant gdiA is schematically shown in Fig. 6A. For the gdiA mutant allele, four different transcripts are observed (Fig. 6B). Out of fourteen individual cDNA clones of RD15.8#16, we found that nine carried an un-spliced intron 2, which would result in a premature STOP codon (TGA) located in intron 2, truncating translation of GdiA at 38 AA. We also found three instances in which intron 2 was successfully spliced, leading to wild type transcripts. Additionally, two cases of alternative splicing were observed: exon 2 skipping (exon 1 joining exon 3) and an alternative splice site in exon 2. Exon 2 (25nt) skipping results in a frameshift that leads to a premature STOP codon (TGA), truncating the protein during translation after 41AA. Interestingly, the alternative splice site found inside exon 2 (GA/GTAGGGA to G/GGAGGGG) results in an in-frame splicing event. This splice variant effectively deletes 5AA (CVLSG) from the 3’ end of exon 2, due an alternative splice site upstream from the wild type exon/intron border. Moreover, because of a new exon 2 boundary, the linkage between exon 2 and exon 3 subsequently yields a single nucleotide change that translates in an E19D substitution.

4. Discussion

This work describes the characterization cell wall chitin mutant RD15.8#16 that was obtained from a previously reported cell wall stress mutant library (Damveld et al., 2008). Disturbance of cell wall integrity has been reported to induce cell wall chitin deposition in filamentous fungi (Fortwendel et al., 2010; Heilmann et al., 2013; Ram et al., 2004; Walker et al., 2015, 2008), and was exploited for a screen to identify high chitin producing strains. RD15.8#16 produces 60% more cell wall chitin than wild type A. niger and, in addition, displays sensitivity to SDS which is known to disturb the cell wall at low concentrations (De Groot et al., 2001; de Nobl et al., 2000; Delley and Hall, 1999). These phenotypes, along with a compact colony morphology, were used in a classical genetics approach by employing co-segregation analysis to identify causative mutations.

For co-segregation analysis, a diploid was obtained from a wild type derivative JN6.2 (ΔnicB::AOpy, ΔolvA::hygB) and mutant TLF54 (a pyrG- and ΔbrnA derivative of RD15.8#16) in a parasexual cross to mix

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Table 2: SNPs located inside open reading frames (ORFs) of TLF54.

| Chromosome | Location | SNP | Gene Description | Gene Orientation | WTSNP | RD15.8#16 SNP | Alteration in Protein |
|------------|----------|-----|-----------------|-----------------|-------|---------------|----------------------|
| chr_1_2    | 1,341,939| NRRL3.01084 | An14g05970 | Multiantimicrobial extrusion family protein | C     | G             | Glu382/504 → Gln382/504 |
| chr_1_2    | 977,103  | NRRL3.01535 | An13g00450 | Hypothetical protein | C     | T             | Leu305/347 → Val305/347 |
| chr_2_1    | 1,969,641| NRRL3.01607 | An01g00340 | Solute carrier family 35 member | C     | CCTT          | Frameshift: Pro255/277 → Arg255/277 |
| chr_2_2    | 977,103  | NRRL3.03466 | An12g03570 | pyrG | Hypothetical protein | G     | GGATC         | Frameshift: Pro255/277 → Arg255/277 |
| chr_4_2    | 2,966,722| NRRL3.05482 | An02g09460 | C2H2-type and FYVE-type zinc finger containing protein (Yeast orthologue PEP7; adaptor protein involved in vesicle-mediated vacuolar protein sorting) | T     | C             | His299/675 → Tyr299/675 |
| chr_4_2    | 2,966,727| NRRL3.06010 | An02g03120 | Rab Gdi family protein | A     | C             | |
| chr_8_2    | 3,040,462| NRRL3.09701 | An11g05410 | Hypothetical protein | A     | G             | |
| chr_8_2    | 3,040,463| NRRL3.11721 | An06g00500 | Hypothetical protein | A     | A             | |

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wild type and mutant chromosomes, followed by subsequent segregation. Co-segregation of the mutant phenotype among the acquired segregants was scored using sensitivity to SDS. Prior to this, we confirmed similar SDS sensitivity of both TLF54 and JN6.2 compared to their respective prototrophic counterparts, RD15.8#16 and RD15.8.

Initial observations on auxotrophic marker segregations suggested equal co-segregation of chromosomes from wild type and mutant, however, unequal segregation of the RD15.8#16 phenotype was observed, resulting in only 2 SDS sensitive segregants from 200 segregants (ΔbrnA#53 and ΔbrnA#90). Low frequency of mutant phenotypes segregation could not be explained by benomyl sensitivity for haploid parents, as RD15.8#16 was affected similarly to benomyl as JN6.2 (Supplementary Fig. 1). Though, certain mutant SNP(s) can be unfavorable during diploid to haploid segregation, either related or unrelated to the SDS sensitivity phenotype. In extension of this observation, we wanted to ensure that there was no disconnect between cell wall chitin and SDS sensitivity for these segregants. This was done by comparing glucosamine content of parental strains JN6.2 and TLF54 with ΔbrnA#53, ΔbrnA#90 and two randomly picked non-SDS sensitive segregants ΔolvA#27 and ΔolvA#41 (Fig. 3B). Results showed that the plate phenotypes (SDS sensitivity and colony morphology) are linked to increased cell wall glucosamine content. SNP co-segregation analysis revealed inherited SNPs from either wild type or mutant in both SDS sensitive and non-SDS sensitive segregants. We were able to identify that ORF-related SNPs recur randomly across segregants, with the exception of SNPs on chromosome IV (Fig. 4), only found in segregants ΔbrnA#53 and ΔbrnA#90.

Chromosome IV located mutant SNPs lay within NRRL3_05482 (Yeast PEP7 orthologue, putative fungal transcription factor) and NRRL3_06010, now identified as gdiA (Rab GDP dissociation inhibitor) based on homology with A. nidulans gdiA (Abenza et al., 2010). Attempts to create single knockouts of both genes was unsuccessful and suggests them to be essential for growth. In congruence with yeast literature, the orthologue of gdiA (GDI1) is a known essential gene (Garrett et al., 1994), whereas PEP7 was previously described as a nonessential gene (Webb et al., 1997). As clean knockout strains could not be obtained, we re-recreated SNPs found in both NRRL3_05482 and gdiA for RD15.8#16 in a wild type background, in order to study their phenotypic effects. Re-creation of SNPs in NRRL3_05482 and gdiA through CRISPR/Cas9 gene editing resulted in viable mutants AG1 and AG3, respectively. Interestingly, only AG3 showed the same level of SDS sensitivity as RD15.8#16, suggesting that the SNPs in the gdiA
mutant allele at the conserved 5′ splice site (+2 and +7 position, G/GTAGGGA to G/GGAGGGG) of the second intron, facilitates both the SD and cell wall chitin phenotype. The data also indicate that the SNP in the NRRL3_05482 mutant allele co-segregated with the gdiA mutant allele as a result of chromosome IV linkage, but is not causative for the RD15.8#16 phenotype.

The T to G SNP in the gdiA mutant allele causes a mutated 5′ donor splice site. The canonical donor-acceptor splice site pair in fungal introns is 5′ GU-AG 3′ (Kupfer et al., 2004). In addition, non-canonical splice sites are observed sporadically being either 5′ GC-AG 3′ or 5′ AU-AC 3′, the former of which represents approximately 90% of non-canonical type splice sites (Burset et al., 2000). This non-canonical splice site was reported to represent 1.2% of all introns in Neurospora crassa with similar ratios for filamentous fungi Fusarium oxysporum and Aspergillus nidulans (Rep et al., 2006). The 5′ GG-AG 3′ type splice sites in intron 2 of the gdiA mutant allele is different from either of these canonical or non-canonical splice sites. Specifically, a G at second position of the 5′ donor splice site (G/G) has previously been shown to result in failure of proper lariat formation for correct transcript processing (Aebi et al., 1987). However, here we show that the 5′ donor splice site in the gdiA mutant allele can result in correct pre-mRNA processing. However, failure of intron 2 splice site recognition was more frequently observed. Interestingly, we also found two cases of alternative splicing: exon skipping and an alternative splice site. The latter variant results in a full-length protein that combines a five amino acids deletion with a single amino acid substitution of glutamic acid to aspartic acid. For now, it remains unknown whether this mRNA yields a functional protein and whether other splice variants may exist. Nevertheless, we have found wild-type mRNA transcripts for gdiA—that are derivative of the mutant allele, suggesting that RD15.8#16 still produces intact, albeit less GdiA. These findings comply with the fact that total loss of gdiA mRNA is lethal.

Rab gdiA encodes a GDP-dissociation inhibitor that functions as a regulator in Rab GTPase cycling. Known orthologues are conserved and have been reported as essential genes in both yeast and Drosophila (Garrett et al., 1994; Ricard et al., 2001). Rab (ras from rat brain (Touchot et al., 1987)) GTPases are involved in regulation of intracellular vesicular transport, continuously cycling between active GTP-bound, and inactive GDP-bound form (Pfeffer, 1992). In the active GTP-bound form, a GTPase is able to interact with downstream effector proteins, assist in cargo selection, transport vesicles, form vesicles from membranes and assist fusion of vesicles with the target membranes (Oesterlin et al., 2014). Once an active GTPase has performed a downstream trafficking cycle at a specific target site, it is subsequently hydrolyzed to a GDP-form. Here, Rab GDI’s can solubilize GDP-bound GTPases from membranes into the cytosol prior to re-deposition at new target membranes. Solubilization by GDIs also prevents turnover of GDP to GTP by GEFs, helping to keep an intracellular steady-state balance of active/inactive GTPases (Pfeffer and Aivazian, 2004; Ullrich et al., 1993). Due to the essential role of GdiA in this balancing act, we propose that a reduction in available GdiA—resultant from inefficient pre-mRNA processing—causes a cytosolic imbalance of soluble GTPases.

Obviously, the essential role that GDP dissociation inhibitors have in cellular processes make them difficult to study their function. In A. niger, gdiA was reported to be repressed by the during exposure of di-thiothreitol (DTT), known to disturb cellular redox homeostasis and trigger the unfolded protein response (MacKenzie et al., 2005). In yeast, a conditional mutant sec19-1 (allelic to GDI1) has been studied and has helped to understand its biological function (Garrett et al., 1994).
Resultant phenotypes include accumulation of ER, Golgi and secretory vesicles as well as defects in protein transport and loss of soluble Rab GTPase Sec4p. Although no cell wall phenotypes have been described for the sec19-1 mutant directly, a conditional mutant of SEC4 (sec4-8), showed random budding patterning, suggesting loss of secretion polarity, an enlarged bud neck and displayed abnormal chitin deposition (Finger and Novick, 1997). Sec4p is an essential protein required for vesicle-mediated exocytic secretion and autophagy (Guo et al., 1999), and relies on the ability to continuously cycle between GTP and GDP, rather than absolute levels of GTP-bound form for proper function (Novick et al., 1993). Unsuccessful release of GDP-bound Sec4p from target membranes as a result of loss or depletion of GDI1 depletes the soluble pool required for re-activation and re- positioning on new target membranes, rendering Sec4p dysfunctional. Consequentially, putative GDP to GTP exchange of old, in situ membrane-bound Sec4p may misallocate protein trafficking, including putative cell wall chitin biosynthesis enzymes. Despite Sec4p and GDI1 involvement in vesicle trafficking, to the best of our knowledge no reports have investigated its role in cell wall biosynthesis or the cell wall integrity pathway.

In A. niger, the closest homologue of Sec4p is secretion related GTPase A (srgA, 58% protein identity) and, unlike in yeast, was found to be a non-essential gene (Punt et al., 2001). The srgA gene was unable to complement a sec4 mutant, suggesting the presence of an additional SEC4 homologue or that SEC4 in yeast governs more vesicle sorting processes than in A. niger. Overall srgA may be related to a more complex multicellular growth behavior (Punt et al., 2001). Interestingly, a deletion of srgA resulted in changes of colonial and peripheral hyphae morphology, similar to RD15.8#16. The compact colony morphology caused by the mutant allele of gdiA in RD15.8#16 may be indirectly related to a regulatory imbalance caused by less GdiA, possibly affecting SrgA cycling. Whether or not there is a relation between srgA and gdiA, we observe an effect of the gdiA mutant allele on cell wall chitin deposition. The fact that A. niger only contains a single genomic copy of a Rab GDI we conclude that less available GdiA as observed in RD15.8#16 affects the Rab GTPase-mediated vesicle trafficking. Consequently, disturbance of these regulatory elements in Rab GTPase cycling most likely causes multiple pleiotropic effects, among which is cell wall chitin biosynthesis.

5. Conclusions

RD15.8#16 was initially isolated in a screen for strains with a continuous state of cell wall stress and, upon further analysis, was found to have increased chitin levels and an increased sensitivity to SDS. Using classical genetics combined with co-segregation analysis and CRISPR/Cas9 gene editing, we showed the involvement of two intronic mutations in gdiA that give rise to this phenotype. It was found that a full gene disruption of gdiA was lethal, and that the observed mutations in the gdiA mutant allele affect intron splicing resulting in reduced levels of functional gdiA transcript. Therefore, we propose that reduced levels of GdiA affect the balance of Rab GTPase cycling. As such, this either influences cell wall chitin deposition directly through increased (ectopic) secretion or indirectly, by general misconstruction (and recycling) of fungal cell wall components that triggers the CWI pathway with a compensatory chitin deposition response. Both hypotheses are not mutually exclusive, but are in congruence with an asymmetrical distribution in GTPase cycling.

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The authors declare no conflict of interest.

Availability of data and materials

The DNA reads described in this study will be deposited in the short read archive upon request. All other data are available on request by contacting the corresponding author.

Appendix A. Supplementary material

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