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cpsA* regulates mycotoxin production, morphogenesis and cell wall biosynthesis in the fungus *Aspergillus nidulans

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Summary

The model fungus *Aspergillus nidulans* synthesizes numerous secondary metabolites, including sterigmatocystin (ST). The production of this toxin is positively controlled by the global regulator *veA*. In the absence of *veA* (ΔveA), ST biosynthesis is blocked. Previously we performed random mutagenesis in a ΔveA strain and identified revertant mutants able to synthesize ST, among them RM1. Complementation of RM1 with a genomic library revealed that the mutation occurred in a gene designated as *cpsA*. While in the ΔveA genetic background *cpsA* deletion restores ST production, in a *veA* wild-type background absence of *cpsA* reduces and delays ST biosynthesis decreasing the expression of ST genes. Furthermore, *cpsA* is also necessary for the production of other secondary metabolites, including penicillin, affecting the expression of PN genes. In addition, *cpsA* is necessary for normal asexual and sexual development. Chemical and microscopy analyses revealed that CpsA is found in cytoplasmic vesicles and it is required for normal cell wall composition and integrity, affecting adhesion capacity and oxidative stress sensitivity. The conservation of *cpsA* in Ascomycetes suggests that *cpsA* homologs might have similar roles in other fungal species.

Keywords

mutagenesis; *veA*; *velvet*; polysaccharide; *cpsA*; mycotoxin; cell wall; development; penicillin; secondary metabolism

Introduction

Aspergillus species synthesize numerous secondary metabolites (Adrio *et al.*, 2003; Brakhage *et al.*, 2011). These compounds include antibiotics and other medical drugs as well as mycotoxins (Bennett *et al.*, 2003; Reverberi *et al.*, 2010). The model fungus *Aspergillus nidulans* produces the mycotoxin sterigmatocystin (ST). This mycotoxin is similar to the well-known potent carcinogenic aflatoxins (AFs) (Payne and Yu, 2010; Sweeny and Dobson,

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1999; Payne and Brown, 1998), produced by other *Aspergillus* species, including the agriculturally important fungi *A. flavus* and *A. parasiticus*. Both toxins are produced through a conserved biosynthetic pathway (Cole and Cox, 1981). As in the case of AF genes, the genes involved in the synthesis of ST are clustered and regulated by *afIR*, an endogenous transcription factor gene (Yu *et al.*, 1996; Keller and Hohn, 1997; Fernandes *et al.*, 1998). Transcription of *afIR*, and concomitant production of ST, is positively controlled by *veA*, a global regulatory gene that also governs sexual and asexual development in *A. nidulans* (Kato *et al.*, 2003; Calvo, 2008; Calvo *et al.*, 2016). A similar role was found in *A. flavus* and *A. parasiticus*, where *veA* controls AF production (Duran *et al.*, 2007; 2009; Calvo *et al.*, 2004).

In *A. nidulans*, the biosynthesis of other natural products, such as penicillin (PN), is also *veA*-regulated (Kato *et al.*, 2003). Similar regulatory output is also observed in other fungal species, for instance, *veA* homologs also regulate PN synthesis in *Penicillium chrysogenum* (Hoff *et al.*, 2010; Veiga *et al.*, 2012; Kopke *et al.*, 2013) and cephalosporin C production in *Acremonium chrysogenum* (Dreyer *et al.*, 2007). Additionally, besides affecting ST and AF production, *veA* also controls the biosynthesis of other mycotoxins, for instance, cyclopiazonic acid and aflatrem in *Aspergillus flavus* (Duran *et al.*, 2007), fumonisins and fusarins in *Fusarium* spp, including *F. verticillioides* and *F. fujikuroi* (Myung *et al.*, 2009; Wiemann *et al.*, 2010), trichothecenes in *F. graminearum* (Merhej *et al.*, 2012), as well as dothistromin in *Dothistroma septosporum* (Chettri *et al.*, 2012) among others.

The use of *A. nidulans* as a model system has greatly enhanced our knowledge on the *veA* regulatory mechanism of action (Calvo *et al.*, 2016). The VeA protein, together with another *velvet* protein denominated VelB (Bayram *et al.*, 2008), is transported to the nucleus by the KapA α -importin, particularly in the dark, a condition that promotes ST production and sexual development in this fungus (Stinnett *et al.*, 2007; Araújo-Bazán *et al.*, 2009). This migration of KapA-VeA-VelB is negatively affected under light by other proteins, such as FphA, LlmF, and Vip-VapB dimer (Palmer *et al.*, 2013; Sarikaya-Bayram *et al.*, 2014; Purschwitz *et al.*, 2008) promoting conidiation. In the nucleus, VeA also interacts with other proteins, forming a complex with light-responsive proteins, FphA, LreA and LreB, which also affects asexual and sexual morphogenesis and secondary metabolism (Purschwitz *et al.*, 2008; Röhrig *et al.*, 2013). Furthermore, VeA also interacts with the putative methyl transferase LaeA (Bayram *et al.*, 2008; Bayram and Braus, 2012) forming the VelB-VeA-LaeA *velvet* complex. LaeA, also required for normal mycotoxin biosynthesis (Bok and Keller, 2004), has been shown to be involved in chromatin remodeling (Reyes-Dominguez *et al.*, 2010; Bayram *et al.*, 2010). Interestingly, not only does VeA play a role as a scaffold for these complexes, but *velvet* proteins have been shown to bind DNA (Beyhan *et al.*, 2013; Ahmed *et al.*, 2013).

Our previous phylogenetic study revealed that VeA is conserved in many Ascomycetes (Myung *et al.*, 2012), where in addition to governing morphogenesis and secondary metabolism, this regulator influences plant pathogenicity of different mycotoxigenic fungi, for example in *Aspergillus flavus* (Duran *et al.*, 2007; 2009), *Fusarium verticillioides* (Myung *et al.*, 2012; Li *et al.*, 2006), *F. fujikuroi* (Bayram *et al.*, 2008), and *F. graminearum* (Merhej *et al.*, 2012; Jiang *et al.*, 2001), as well as fungal infections in animals (Laskowski-

Peak *et al.*, 2012). Absence of *veA* homologs in these fungi resulted in a decrease in virulence, coinciding with a reduction in mycotoxin production. In addition, cellular processes such as oxidative stress response (Calvo *et al.*, 2016; Baidya *et al.*, 2014), cell wall structure synthesis (Li *et al.*, 2006; Park *et al.*, 2015), and presence of hydrolytic activity (Dhingra *et al.*, 2012; Duran *et al.*, 2014) have all been found to be modulated by *veA*.

In order to discover novel genetic elements connected to the *veA* global regulatory mechanism we previously performed random mutagenesis in a deletion *veA* strain, and identified several *veA*-dependent genes involved in the regulation of ST biosynthesis (Ramamoorthy *et al.*, 2012). For instance, *rtfA*, a gene encoding a putative RNA polymerase transcription elongation factor-like that also regulates sexual and asexual development. In the same manner, we found *mtfA*, encoding a novel putative C₂H₂ zinc finger domain type transcription factor that regulates the biosynthesis of several secondary metabolites including ST, and it is also important for morphogenesis in *A. nidulans* (Ramamoorthy *et al.*, 2013). In the present study we have investigated another selected revertant mutant, RM1. This strain presented a point mutation in a gene that we designate as *cpsA*. This gene was initially annotated as encoding a putative polysaccharide synthase. In this study we show that *cpsA* differentially influences ST biosynthesis in a *veA*-dependent manner. *cpsA* also regulates the biosynthesis of other natural products, including PN. Furthermore, *cpsA* is necessary for normal conidiation and sexual development in *A. nidulans*. Importantly, our chemical analysis and microscopy observations indicated that *cpsA* is required for normal composition and integrity of the fungal cell wall, affecting fungal adhesion and the capacity to survive under exposure to environmental stresses. Our study demonstrated a profound effect of *cpsA* on the expression of numerous genes, including those involved in secondary metabolism and developmental processes.

Results

AN9069 annotated as a putative polysaccharide synthase

Previously we reported seven revertant mutants (RMs) with restored wild-type levels of norsolorinic acid (NOR), an orange intermediate used as marker of ST biosynthesis, in a strain with a *veA* background (Ramamoorthy *et al.*, 2012). In the present study we identified the mutated gene in RM1 (Figure 1). First, to examine whether one or more mutations occurred in RM1, the strain was crossed with RJH030 (*yA2*, *wA3*, *pabaA1*, *stcE*, *trpC801*, *veA1*). Analysis of the obtained progeny and dominant analysis revealed that RM1 presents a single recessive mutation. Transformation of RM1-R2 with a genomic library yielded colonies with wild-type phenotype. Sequencing of the inserts in rescued plasmids from these fungal colonies, and comparison of these sequences with the *A. nidulans* genomic database (<http://www.aspgd.org>) by BLAST analysis showed that the mutated gene in RM1 and RM1-R2 strains was AN9069, annotated as a putative polysaccharide synthase gene, and was denominated *cpsA*. The mutation was a G-A transition at nucleotide +568 of the *cpsA* coding region, changing the codon from GAT to AAT, resulting in a substitution of glutamic acid (D) for asparagine (N) (Figure S1). Complementation of RM1 with the library plasmid containing *cpsA* resulted in a strain with *veA* phenotype with reduced ST intermediate production (Figure 1).

***cpsA* putative homologs are present in other fungal species**

The deduced amino acid sequence of *A. nidulans* CpsA revealed high identity with proteins from other *Aspergillus* species, including *A. clavatus* (75.4% identity), *A. terreus* (78.7%), *A. flavus* (76.5%), or *A. fumigatus* (65.3%) (Figure S1, Table S1). TMHMM predicted hydrophobic regions possibly acting as signal peptide (1–36, probability below 0.4) and transmembrane domains TM1 (309–332, probability below 0.4), TM2 (343–367, probability below 0.6) and TM3 (371–397, probability below 0.9) (Figure S1).

Analysis of other fungal genome databases revealed that CpsA is also conserved in many other Ascomycetes and also in Basidiomycetes (Table S1). An extensive alignment and phylogenetic tree is shown in Figures S2 and S3.

Additionally, *A. nidulans* CpsA was compared with putative bacterial homologs (Figure S4). CpsA presents a DXD motif, conserved in glycosyltransferases (Breton et al., 2006), that is relevant for their activity (Bothe et al. 2015; Busch et al., 1998; Li et al; 2001). This DXD motif was shown in crystal structures to interact primarily with the phosphate groups of a nucleotide donor through the coordination of a divalent cation, typically Mn^{2+} (Breton et al., 2006) or Mg^{+2} (Weigle, 2015). In addition, *A. nidulans* CpsA contains the related XDD motif (Weigel, 2015; Gulberti, et al., 2003).

***cpsA* affects mycotoxin biosynthesis in a *veA*-dependent manner**

To further evaluate the effects of *cpsA* on *A. nidulans* mycotoxin biosynthesis, we deleted the *cpsA* coding region in the RDAE206 strain (*veA*, *stcE*), creating TDAE *cpsA* (*cpsA*, *veA*, *stcE*) (Figure S5) (Table S2). In addition, we also deleted *cpsA* in a strain with a *veA*⁺ wild-type allele, RJMP1.49, resulting in TRV *cpsA* (*cpsA*, *veA*⁺) (Table S2) (Figure S5). These deletions were confirmed by Southern blot analysis (Figure S5). The deletion of *cpsA* in a *veA* *stcE* background shows similar NOR production levels to those in *cpsA*- *veA* *stcE* and the control *veA*⁺ *stcE*, and higher than in *veA* *stcE* (Figure 1). However, deletion of *cpsA* in a strain with a *veA*⁺ genetic background, showed a delay and a reduction in ST production compared with its isogenic controls strain, and the complementation strain (Figure 2). The reintroduction of the wild-type allele partially rescues the phenotype close to wild-type levels. It is possible that the complementation occurred at a different location, resulting in the observed slight phenotypical changes.

ST production is affected by glucose concentration (i.e. Atoui et al., 2010). For this reason we also evaluated the effect of *cpsA* on mycotoxin production on medium with different glucose concentrations. *cpsA* *veA*⁺ strain also showed a delay and a reduction in ST production when medium with higher concentrations of glucose was used (Figure S6). Overexpression of *cpsA* also showed a reduction in ST biosynthesis with respect to the controls. This difference in mycotoxin production was more notable as the concentration of glucose in the medium was increased. In addition, our analysis revealed that the synthesis of other unknown compounds was also *cpsA*-dependent (Figure S6).

The genes responsible for ST production are located in a cluster (Brown et al., 1996), among them, *affR* encodes a C6 zinc transcription factor that activates other genes in this cluster (Yu et al., 1996; Fernandes et al., 1998; Brown et al., 1996). Additionally, another gene

within the ST cluster, *stcU*, that encodes a ketoreductase, is commonly used as indicator of ST cluster activation (Yu *et al.*, 1996; kato *et al.*, 2003; Keller *et al.*, 1995; Hicks *et al.*, 1997). Northern analysis showed that expression of both *afIR* and *stcU* was reduced in the *cpsA* deletion strain (TRVp *cpsA*) (Table S2) in comparison to the isogenic wild type and complementation strains (Figure 2A). The same trend was also observed when *cpsA* was overexpressed. A concomitant reduction in ST accumulation was detected in the deletion and overexpression *cpsA* cultures (Figure 2B).

PN production is influenced by *cpsA*

Our chemical analysis results indicated that *cpsA* also affects the synthesis of other compounds in addition to ST (Figure S6). It is known that production of several secondary metabolites, including ST and PN, can be coregulated by certain proteins, such as VeA (Kato *et al.*, 2003), and that, based on our current study, the *cpsA* genetic element is VeA-dependent. For these reasons, we also evaluated whether *cpsA* influences PN production in *A. nidulans*. We analyzed production of this antibiotic in the *cpsA* strain (TRVp *cpsA*) and compared it with PN levels in the control strains, as well as in the *cpsA* overexpression strain. For this assay we used a well-established bioassay using *B. calidolactis* as the testing organism. Absence or overexpression of *cpsA* significantly decreased PN production with respect to the control strains (Figure 3), indicating that normal *cpsA* expression is necessary for wild-type levels of PN production. Furthermore, expression of the genes involved in PN biosynthesis, *acvA*, *ipnA* and *attA* (MacCabe *et al.*, 1990), was significantly down-regulated when *cpsA* is deleted or overexpressed, causing reduction in PN biosynthesis.

cpsA positively affects development in *A. nidulans*

Deletion of *cpsA* resulted in a reduction in colony growth (Figure 4) and a drastic alteration of *A. nidulans* morphology (Figure 5), affecting both asexual and sexual development. Absence of *cpsA* resulted in severe reduction of conidial production, as well as in loss of Hülle cells (nursing cells contributing to the formation of fruiting bodies), and lack of cleistothecial formation under conditions that allow the production of these structures in the isogenic controls (Figure 5A–B). Interestingly, overexpression of *cpsA* also decreased conidiation, while causing an increase in the number of Hülle cells and a slight increase in cleistothecia with respect to the controls (Figure 5A–B). The reduction in conidiation in *cpsA* and OE*cpsA* was accompanied by a decrease in the expression of *brlA*, a transcription factor gene that regulates early stages in conidiophore formation (Figure 5C) (Boylan *et al.*, 1987; Adams *et al.*, 1988). This reduction in *brlA* expression was particularly notable in *cpsA*. In addition, the lack of sexual development in the absence of *cpsA* also coincided with a decrease in the expression levels of *nsdD* and *steA*, both encoding transcription factors involved in sexual development (Han *et al.*, 2001; Vallim *et al.*, 2000). Furthermore, deletion of *cpsA* negatively affected the expression of *veA* (Figure 6), global regulator previously described to be essential for sexual development and other biological functions (Kato *et al.*, 2003; Calvo *et al.*, 2016; Yager, 1992; Kim *et al.*, 2002; Calvo and Cary, 2014), as well as *laeA*, encoding a VeA-interacting partner, LaeA, that also influences sexual development and controls secondary metabolism (Bok and Keller, 2004; Bayram *et al.*, 2010).

CpsA localizes in cytoplasmic vesicles

In the current study we also investigated the subcellular localization of CpsA in *A. nidulans*. With this purpose, we generated two identical strains with *cpsA* fused to *gfp*. A single copy of the fusion cassette was detected at the *cpsA* locus (data not shown). The selected transformants containing the *cpsA::gfp* cassette presented wild-type phenotype. Microscopic observations were identical for both transformants (results for TXF1.1 are shown in Figure 7). Fluorescence microscopy results indicated *A. nidulans* CpsA-GFP localizes abundantly at cytoplasmic vesicles or organelles. CMAC staining indicates that some of these organelles are vacuoles. Non motile and other highly motile vesicles are likely endosomes (Movie 1). No other structures are labeled by GFP fluorescence. For example there is no evidence of accumulation at the plasma membrane. Over-expression of CpsA does not modify this distribution, only the intensity of fluorescence detected (Figure S7, Movies 2 and 3). Thus CpsA might be exerting its role in any of those vesicles, or the amount of protein at the plasma membrane is relatively low for detection. Full length CpsA-GFP fusion protein was detected by Western blot. This protein must be linked to structures that cannot be solubilized in a standard buffer lacking detergents or denaturing agents (A50 buffer, see extensive work with this buffer in Orejas et al., 1995) corroborating the microscopy result of localization in internal membranous structures. Nevertheless, the GFP moiety is detected suggesting a degradation process for CpsA-GFP probably at recycling compartments as the lysosome or multivesicular bodies.

cpsA is involved in regulating cell wall composition and integrity

Our sequence analysis suggested that *cpsA* could be involved in cell wall organization or synthesis. To test whether *cpsA* has a role in cell wall integrity, we examined the effect of cell wall disrupting compounds, sodium dodecyl sulphate (SDS) and calcofluor white (CFW) (Ram and Klis, 2006) in the culture medium inoculated with the wild type, *cpsA*, *cpsA-com*, and *OEcpsA* (Figure 8). In both cases the presence of the cell wall disrupting agent caused growth inhibition when *cpsA* was deleted or over-expressed. The observed effect was particularly drastic in the absence of *cpsA*. These results suggest that *cpsA* might affect cell wall components. We examined the cell wall composition in the wild type, *cpsA* deletion, complementation and overexpression strains as detailed in Materials and Methods. The results showed that absence of *cpsA* led to a significant reduction of glucan, chitin and mannoprotein compared to the levels of those components in the wild-type and complementation strain cell wall (Table 1). Overexpression of *cpsA* resulted in a similar reduction of these components compared to the controls. Importantly, transmission electron microscopy analysis revealed that absence of *cpsA* results in partial detachment of the cell wall from the plasma membrane (Figure 9). This observed separation was consistent only in the case of the *cpsA* mutant, while it was not observed in the control strains.

cpsA is involved in adhesion to surfaces

Biofilms are complex structures that offer protection from environmental stresses. Biofilm formation has been shown to involve a sequential adherent process to a new surface (Finkel and Mitchell, 2011; Beauvais and Muller, 2009). We examined the possible role of *cpsA* in *A. nidulans* mycelial adhesion. Interestingly, our results indicated that deletion of *cpsA*

caused a notable delay and reduction in adhesion capacity (Figure 10). Overexpression of *cpsA* also slightly decreased adhesion capacity at 24 h after inoculation. In addition, we analyzed the expression of three genes that could influence biofilm formation, *dvrA*, *stuA* and *laeA* (Figure 10B). In all cases these three genes were downregulated in the *cpsA* deletion strain compared to the controls.

Wild-type *cpsA* expression levels facilitate *A. nidulans* growth under oxidative stress conditions

In order to examine the possible role of *cpsA* in resistance to oxidative stress, menadione was added to the culture medium at different concentrations (Figure 11A). Absence of *cpsA* clearly resulted in growth inhibition at 0.08 mM, while the growth of the isogenic control strains was inhibited at 0.2 mM under the experimental conditions assayed. Overexpression of *cpsA* only mildly decreased fungal growth under oxidative stress conditions. Furthermore, alterations in the *cpsA* locus reduced the expression of the thioredoxin gene *thiO* and catalase gene *catB* with respect to the controls when the cultures were exposed to menadione (Figure 11B–D).

Deletion of *cpsA* affects osmotic stress tolerance and increases sensitivity to temperature in *A. nidulans*

In this study, we also examined whether *cpsA* was involved in survival under osmotic stress. For this purpose the medium was supplemented with NaCl (0.7 M), or sorbitol (1.2 M). The addition of sorbitol did not further decrease fungal growth in the deletion mutant (Figure S8). However, addition of NaCl did reduce *cpsA* colony growth in comparison to the colonies of this strain grown on non-supplemented GMM. Absence of *cpsA* also resulted in an increase in sensibility to higher temperatures (45 °C), while a lower temperature (30 °C) increased fungal growth in this mutant. Unexpectedly, NaCl and sorbitol supplemented *cpsA* cultures grown at 45 °C showed an increase of vegetative growth.

Defects in cell wall are not sufficient to recover ST production in a *veA* background

In order to test whether the defects in cell wall composition and integrity observed in the *cpsA* mutant were sufficient to recover ST biosynthesis in a strain with a *veA* background, we analyzed production of this mycotoxin in the double mutant *veA rlmA*. The *rlmA* gene encodes a MADS-box transcription factor necessary for cell wall integrity in *A. nidulans* (Kovács et al., 2013). Our results indicate that alterations in the *rlmA* locus are insufficient to reestablish toxin production in the absence of *veA* (Figure S9).

Analysis of hyaluronic acid by SAX-HPLC and glycosyl composition of cell wall by GC-MS

In order to determine whether hyaluronic acid is present or absent in *A. nidulans* cell wall and whether its synthesis is *cpsA*-dependent, we examined hyaluronan content in these samples at the Complex Carbohydrate Research Center (Athens, GA). After digestion with hyaluronase, samples were analyzed by SAX-HPLC as described in the Materials and Methods section, and compared to a purified hyaluronan sample undergoing the same procedure. Our results showed that hyaluronan was not detected in these fractions. In addition, monosaccharide composition of the wild-type and *cpsA* cell wall fractions was

also analyzed by GC-MS. Hyaluronan is a polymer of D-glucuronic acid and D-N-acetylglucosamine, however no glucuronic acid was found in these fractions (Table S3), further indicating that hyaluronan is absent or at very low concentration in the cell wall of this fungus.

Analysis of exopolysaccharide (EPS) glycosyl composition by GC-MS

In order to examine whether hyaluronan is present in *A. nidulans* EPS, we analyzed its glycosyl composition by GC-MS (Table S4). As in the case of the cell wall analysis, no glucuronic acid was found in the EPS of either wild type or mutant. In addition, EPS was analyzed by infrared spectroscopy (Figure S10). IR spectra from both samples were identical and presented characteristics of polysaccharides with N-acetylated aminosugars. The bands at 820 and 850 cm^{-1} and the lack of signal at 890 cm^{-1} confirmed the alpha configuration of these polysaccharides. Furthermore, the signals at 1558 and 1646 cm^{-1} are typical of the amide linkage from N-acetyl aminosugars, which suggest N-acetylation of galactosamine units. The absence of signals in the region of 1700–1780 cm^{-1} from the carboxyl group confirms that these samples lack uronic acids, which agrees with the results from monosaccharide analysis. The type of linkages was also analyzed after methylation (Figure S11). The results from the methylation analysis as well as those from IR spectroscopy indicate the presence of α -(1–4)-galactosaminogalactans in the EPS samples.

Analysis of monosaccharides in *A. nidulans* membrane

As mentioned above, hyaluronan is a polymer of D-glucuronic acid and D-N-acetylglucosamine. We analyzed the monosaccharide composition of *A. nidulans* wild-type membrane searching for any of these monomers, particularly glucuronic acid, which could serve as possible anchor between membrane and cell wall by binding D-N-acetylglucosamine in that structure. Furthermore, glucuronic acid containing glycolipids have been reported in membranes of the phylogenetically related fungus *A. fumigatus* (Fontaine et al., 2009). Our GC/MS analysis results are listed in Table S5. Mannitol, mannose, glucose, galactose and glucosamine were detected, however glucuronic acid was not found in this sample.

Discussion

Secondary metabolites are crucial in the ecology and adaptation of fungi to their environment. The biosynthesis of these compounds, also denominated natural products, is often genetically linked with morphological development by shared regulatory mechanisms (Calvo, 2008; Calvo et al., 2016; Bayram and Braus, 2012; Calvo and Cary, 2014; Calvo et al., 2002; Yu and Keller, 2005). Among them, one member of the *velvet* protein family, VeA, plays a key role in connecting these two processes (Calvo, 2008; Calvo et al., 2016; Bayram and Braus, 2012; Calvo and Cary, 2014; Calvo et al., 2002). In *A. nidulans*, the absence of the *veA* gene results in strains unable to form sexual fruiting bodies (Kim et al., 2002), displaying hyperconidiation by affecting the expression of *brlA*, a gene encoding an indispensable transcription factor for the formation of conidiophores in this model fungus (Kato et al., 2003). In other *Aspergillus* species, such as the AF-producers *A. flavus* and *A. parasiticus*, deletion of *veA* prevents the formation of sclerotia (Calvo et al., 2004; Duran et

al., 2007; Calvo and Cary, 2014), resistant structures important for survival under adverse environmental conditions (Coley-Smith and Cooke, 1971; Wicklow, 1987). It has been established that *veA* has a plurality of roles. Recent transcriptome analyses revealed a wide range of *veA*-dependent genes with a variety of functions, including numerous secondary metabolite gene clusters in *A. nidulans*, as well as in other *Aspergillus* species, such as the opportunistic human pathogen *A. fumigatus* (Dhingra *et al.*, 2013; Lind *et al.*, 2015) and the AF-producer *A. flavus* (Cary *et al.*, 2015), as well as in other fungal genera, for example in *Fusarium* (Wiemann *et al.*, 2010; Kim *et al.*, 2013) or in *Penicillium* spp. (Hoff *et al.*, 2010). While the compounds associated with some of these *veA*-dependent genes clusters are still unknown, in some cases these associations have been characterized in great depth, such as the case of the mycotoxin ST in *A. nidulans*.

In spite of its importance, the VeA mechanism of action is not fully understood. Our search for new *veA*-dependent genetic elements involved in the regulation of ST production in *A. nidulans* resulted in the identification of *cpsA*, encoding a protein initially annotated as a putative polysaccharide synthase. CpsA shares limited similarity with proteins of the bi-functional glycosyltransferases family 2, hyaluronan synthases, which catalyze polymerization of hyaluronan. The deduced amino acid sequence shows a putative signal peptide domain and three putative transmembrane domains. Although the probability in this prediction is low, these domains have also been described in other characterized hyaluronan synthases, including those in humans (Watanabe and Yamaguchi, 1996). Its putative characterized bacterial homolog, Cps1, is a type 3 polysaccharide synthase in *Streptococcus pneumoniae*, involved in the polymerization of Glc and GlcUA onto the growing polysaccharide chain (Chang *et al.*, 2006). In the pathogenic yeast *Cryptococcus neoformans*, the Cps1 homolog is a hyaluronic acid synthase needed for the formation of the outer cell wall, playing an essential role in adhesion and virulence of this Basidiomycete (Chang *et al.*, 2006; Jong *et al.*, 2007). A Cps1 putative homolog was also found in *Neurospora crassa* by Fu *et al.* (2014). As in the case of *N. crassa*, hyaluronic acid was not detected in our analysis of *A. nidulans* cell wall. Furthermore, our extensive chemical analysis indicates that this compound is also absent in the exopolysaccharide, mainly composed of α -(1-4)-galactosaminogalactans. In addition, no evidence of glucuronic acid was found in membrane. Nevertheless, our comparative analysis with putative bacterial homologs indicated the presence of DXD and XDD motifs, conserved in 78 families of glycosyltransferases (Breton *et al.*, 2006; Bothe *et al.* 2015; Busch *et al.*, 1998; Li *et al.*; 2001). It is possible that *cpsA* could influence carbohydrate metabolism, which is known to affect ST production (i.e. Németh *et al.*, 2016). Irrefutably, our results revealed a profound effect of *cpsA* in toxin production as well as in many other aspects of *A. nidulans* biology. For this reason we also performed a detailed microscopy study to provide additional clues of its mode of action. Our results revealed that CpsA-GFP accumulates in cytoplasmic vesicles or organelles and that, based on the CMAC staining, some of these organelles are vacuoles. Non motile vesicles would correspond to late-endosomes, and those moving in retrograde and anterograde directions are, most probably, early endosomes (Lopez-Berges *et al.*, 2016). Additionally, our protein analysis also indicates that CpsA is located and bound to internal membranous structures. Functional full-length CpsA-GFP protein must locate in early and late endosomes, or other membranous compartment, such as vesicles originated at the Golgi

which could fuse to early endosomes. The recycling of this putative transmembrane protein probably occurs in vacuoles or multivesicular bodies fused with lysosomes, as also suggested by the presence of the resistant GFP fragment in the Western Blot. The vesicle transport machinery has been shown to be involved in the synthesis, compartmentation and export of aflatoxin in *A. parasiticus* (Chanda et al., 2009). Furthermore, the formation of these vesicles (aflatoxisomes) is at least in part mediated by *veA*. It is possible that *cpsA* could be involved in processes affecting the synthesis of ST in similar vesicles in *A. nidulans*. Future research will be carried out in our laboratory to investigate the nature of these membranous compartment and their aggregates.

Our current study also revealed that CpsA influences ST biosynthesis in a *veA*-dependent manner in *A. nidulans*. Deletion of *cpsA* in a strain with a *veA*⁺ allele resulted in the opposite effect on mycotoxin production with respect to that observed in a strain where *veA* is absent, presenting a decrease in accumulation of ST in comparison with the wild type. Similarly to the case of strains with a deletion *veA* background, strains with a *veAI* partially functional mutant allele (Stinnett et al., 2007; Kim et al., 2002; Käfer, 1965) (commonly used in *A. nidulans* research laboratories), with a loss-of-function *cpsA* allele also accumulated an abundant amount of ST. These results indicated that *cpsA* function is affected by the *veA* allele. This effect on ST production was also observed in previously characterized *veA*-dependent genes, such as *rtfA* (Ramamoorthy et al., 2012), and *mtfA* (Ramamoorthy et al., 2013). Furthermore, our study also showed that *cpsA* affects the expression of *afIR*, encoding a C6 zinc transcription activator necessary for the activation of other genes in the ST gene cluster (Yu et al., 1996; Fernandes et al., 1998). The biosynthesis of other secondary metabolites was also impacted by the presence or absence of *cpsA*, for instance, production of PN was reduced in the *cpsA* deletion mutant as well as in a strain with forced overexpression of this gene. This suggests a broad effect of *cpsA* on *A. nidulans* secondary metabolism, in which proper wild-type levels of *cpsA* expression are necessary for normal biosynthesis of these natural products.

Besides the observed effect of *cpsA* in secondary metabolism, the present study indicated that *cpsA* also affected *A. nidulans* development. Expression of *brlA*, key in the conidiation central regulatory pathway (Boylan et al., 1987; Adams et al., 1988; Park and Yu 2012; Adams and Yu, 1998) was downregulated in the absence of *cpsA* compared to the wild type, resulting in a reduction of conidial production. Furthermore, in the *cpsA* deletion mutant, expression was also decreased for the *nsdD*, *steA*, and *stuA* genes, which encode transcription factors required for sexual development (Han et al., 2001; Vallim et al., 2000; Wu and Miller, 1997; Miller et al., 1992). Furthermore, the *cpsA* deletion strain is unable to produce Hülle cells or cleistothecia under conditions that allow the production of these structures in the wild type. As in the case of *A. nidulans*, loss of the CpsA homolog, Csp-1, negatively affected conidiation and production of sexual structures in *N. crassa* (Fu et al., 2014). Overexpression of *cpsA* in *A. nidulans* resulted in greater production of Hülle cells and cleistothecia than the wild type. These results indicate that *cpsA* positively influences both asexual and sexual development in *A. nidulans*.

Interestingly, this study showed that not only is *cpsA* functionally *veA*-dependent, but that *cpsA* also positively affects the expression of the global regulators *veA* and *laeA*; the later

encodes a putative methyl transferase involved in chromatin conformation remodeling (Bok and Keller, 2004; Reyes-Dominguez *et al.*, 2010; Bayram *et al.*, 2010; Patananan *et al.*, 2013). Since both *veA* and *laeA* regulate hundreds of genes in *A. nidulans* (Lind *et al.*, 2015; Kim *et al.*, 2013a) as well as in other fungi (i.e. Hoff *et al.*, 2010; Wiemann *et al.*, 2010; Dhingra *et al.*, 2012; Lind *et al.*, 2015; Cary *et al.*, 2015; Kim *et al.*, 2013a; Karimi-Aghcheh *et al.*, 2013; Fekete *et al.*, 2014) it is possible that some of the phenotypes observed in the absence of *cpsA*, such as changes in morphological and chemical development, could be, at least in part, the result of alterations in the expression of these global regulators (Figure 12). Moreover, *veA* transcriptome analysis in *A. nidulans* indicated that *cpsA* expression is also dependent on *veA* (Lind *et al.*, 2015), suggesting a regulatory loop essential for *A. nidulans* fitness.

The absence of *cpsA* was also associated with defects in cell wall composition, including a decrease in glucan, chitin and mannoprotein content. Moreover, transmission electron microscopy analysis revealed alterations in cell wall integrity with structural changes in the *cpsA* deletion mutant, presenting partial detachment of the cell wall with respect to the plasma membrane. It is possible that this partial detachment could be a consequence of a disorganized assembly of the cell wall. Additionally, it is likely that some of the cell wall components could be more easily lost in the *cpsA* mutant strain, as in the case of cell wall proteins in the *N. crassa* Cps1 mutant (Fu *et al.*, 2014). It has been shown that deletion of a gene involved in cell wall biosynthesis may induce a compensatory effect further modifying the structure (Beauvais *et al.*, 2014; Latgé, 2010). However, our results suggest that the lack of *cpsA* does not lead to a compensatory effect in this case. The reduction of other cell wall components and structural alterations in the *cpsA* mutant cell wall could contribute to the observed increase in sensitivity to external stresses. For example, the fungal cell wall is the first line of defense for protection against exogenous oxidative stress (Fuchs and Mylonakis, 2009). Our results indicated that alterations in the expression of *cpsA* increases sensitivity to reactive oxygen species (ROS). In addition, wild-type expression levels of *cpsA* are also necessary for proper expression of *thiO*, *catB* (Hong *et al.*, 2013; Kawasaki and Aguirre, 2001) and *veA*, also known to be involved in oxidative stress response (Baidya *et al.*, 2014), that further contributes to this increased sensitivity to ROS in strains without a wild-type *cpsA* locus. Oxidative stress influences activation of fungal development and secondary metabolism (i.e. Baidya *et al.*, 2014; Etxebeste *et al.*, 2010; Amare and Keller, 2014; Roze *et al.*, 2013). However above certain thresholds, excessive exposure to ROS and other stresses in the *cpsA* mutant could lead to deleterious effects, negatively affecting the expression of numerous genes, including those involved in sexual and asexual development, as well as those involved in secondary metabolism in *A. nidulans*. Interestingly, in the case of ST, deletion of the *rlmA* gene, involved in cell wall integrity (Kovács *et al.*, 2013) in a *veA* strain did not recover toxin production, suggesting that although *cpsA* notably affects *A. nidulans* cell wall, this effect does not appear to be solely responsible for some of the biological changes observed in the absence of *cpsA*.

It is known though that the fungal cell wall not only plays an essential role in maintaining cell shape and protecting the cell against mechanical damage and other environmental stresses, but it is also involved in adhesion to environmental surfaces and in biofilm formation (de Groot *et al.*, 2009; Yin *et al.*, 2008; Brown *et al.*, 2014). In pathogenic fungi,

biofilms cause clinical problems, including resistance to antifungal drugs (Ramage *et al.*, 2009). Importantly, our results revealed that *cpsA* is necessary for normal adhesion to surfaces. Biofilm formation, for which adhesion is necessary, is associated with complex regulation of cell surface protein synthesis, hyphal formation and secondary metabolism (Finkel and Mitchell, 2011; Beauvais *et al.*, 2014). Physical contact, for example contact of fungal mycelium with bacteria, has been shown to be required to elicit a response that triggers the expression of specific secondary metabolite gene clusters (Schroeckh *et al.*, 2009). This evidence supports that adhesion, which allows proper physical contact with surfaces, is intimately related with secondary metabolism regulation in response to this environment perception. In addition, in *A. fumigatus*, the secondary metabolite regulator *laeA*, was found highly upregulated during biofilm formation (Gibbons *et al.*, 2011). In our studies, lack of proper adhesion in the *cpsA* mutant formation coincided with a reduction of *laeA* transcription, which could also negatively affect secondary metabolism. The reduction in adhesion in the absence of *cpsA* was also associated with a decrease in the expression of the developmental gene *stuA*, homolog of *Candida albicans efg1*, which is necessary for biofilm formation in this pathogenic fungus (Finkel and Mitchell, 2011; Gibbons *et al.*, 2014). *stuA* plays multiple roles in fungi; in *A. nidulans*, besides its described role in the regulation of sexual development and possibly in adhesion, as suggested in our study, it is also involved in the regulation of asexual development (Wu and Miller, 1997; Miller *et al.*, 1992), this holds true in *A. fumigatus* (Sheppard *et al.*, 2005), as well as its role governing some secondary metabolite gene clusters (Sheppard *et al.*, 2005; Twumasi-Boateng *et al.*, 2009). In addition, the multiple functions described for *stuA* include the regulation of catalase-peroxidase gene *cpeA* in *A. nidulans* (Scherer *et al.*, 2002), as well as *cat1* in *A. fumigatus* (Sheppard *et al.*, 2005). It is possible that besides the effects observed in development and possibly in biofilm formation, the reduction of *stuA* in the *cpsA* mutant could further contribute to accentuate the increased sensitivity to ROS. Lastly, in our study, loss of adhesion capacity in *cpsA* was also associated with a reduction in the expression of the *A. nidulans* homolog of the *A. fumigatus* C2H2 zinc finger transcription factor gene *dvrA* (Ejzykowicz *et al.*, 2010), and *C. albicans* and *C. parasilosis bcr1* (Fanning and Mitchell, 2012; Nobile and Mitchell, 2005; Ding and Butler, 2007), known to play a critical role in biofilm formation.

In conclusion, our studies revealed that the *veA*-dependent genetic element *cpsA* in *A. nidulans* has a profound effect on secondary metabolism and morphological development. *cpsA* also proved to be critical for proper cell wall composition and integrity, as well as for normal adhesion capacity. The critical role of *cpsA* in fungal cell wall formation could in part determine resistance to environmental stressors, such as presence of ROS, osmotic stress or temperature variation. These multiple direct or indirect effects suggest a cross-talk and complex balance between different *cpsA*-dependent cell processes in *A. nidulans* that could be in part mediated by the effect of *cpsA* on the global regulators *veA* and *laeA*. The fact that the absence of *cpsA* results in a range of dramatic defects in this filamentous model fungus, together with the conservation of *cpsA* in many other fungal species, makes *cpsA* a potential desirable target in a control strategy to reduce the detrimental effects of fungi, including mycotoxin contamination. Additionally, since *cpsA* homologs are present in multiple fungal pathogens, this gene and its gene product could also be used as target for

antifungal drugs. The study of *A. nidulans cpsA* exemplifies the strong interdependence of different cellular processes necessary for fitness and survival of fungal species.

Experimental Procedures

Fungal strains and growth conditions

The *A. nidulans* strains used in this study are listed in Table S2. Strains were grown on glucose minimum medium (GMM) (Käfer, 1977) and oat meal medium (OMM) (Butchko *et al.*, 1999) containing the appropriate supplements for the corresponding auxotrophic markers (Käfer, 1977). Ten grams per liter was added in the case of solid medium. Strains were stored as 30% glycerol stocks at -80°C .

Genetic techniques

Meiotic recombination was established between *A. nidulans* RM1 mutant obtained by chemical mutagenesis (Ramamoorthy *et al.*, 2012) and the RJH030 strain (*pabaA1*; *yA2*; *wA3*; *argB2*; *stcE::argB*; *trpC801*; *veA1*) as previously described (Pontecorvo *et al.*, 1953). The progeny was analyzed for the presence or absence of the *veA1* allele, first by phenotypic observation and finally confirmed by PCR. Colony morphology, as well as norsolorinic acid (NOR) production, were also examined. The progeny of this cross showed four phenotypic groups: 1. *veA*, *stcE*, X- (RM1 parental type); 2. *stcE* (RJH030 parental type); 3. recombinant type *veA*, *stcE* (deletion *veA* phenotype); 4. recombinant type *stcE*, X- (RM1-R2). Dominance test was carried out by generating diploids using RM1-R2 and RAV1 strains. Diploids were analyzed for NOR accumulation as well as morphological development.

Identification of the revertant mutation in RM1

To identify the mutated gene originally present in RM1, the RM1-R2 (*stcE*, X-) strain was transformed with the *A. nidulans* genomic library pRG3-AMA1-NOT1. Plasmid DNA was extracted from fungal transformants showing wild-type phenotype. The end regions of the DNA inserts in the obtained plasmids were sequenced. The complete DNA insert sequence was found in the *A. nidulans* genome database (<http://www.aspgd.org>). To elucidate the location of the mutation in RM1, a PCR product was amplified from the same locus in RM1 and sequenced.

Sequence analysis

The deduced amino acid sequence of *cpsA* (AN9069) was compared against databases from other *Aspergillus* species and from different fungal genera, using the BLAST (blastp) tool provided by National Center for Biotechnology Information (NCBI), (<http://www.ncbi.nlm.nih.gov/>). TMHMM website (<http://www.cbs.dtu.dk/services/TMHMM/>) was also used for prediction of hydrophobic regions possibly acting as signal peptide or transmembrane domains in the comparison between several CpsA homologs from different *Aspergillus* species. The gene entry with the highest percentage of identity and the lowest e-value for each of the species was selected. Pairwise sequence alignment of the proteins was performed using the EMBOSS Needle tool (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) from EMBL-EBI (European Molecular Biology Laboratory's European

Bioinformatics Institute). Percentage of similarities and percentage of identities were tabulated for each of the alignments. Multiple sequence alignment was performed with CpsA (*A. nidulans*) and homologs found across various fungal species using MAFFT version 6.0 (<http://mafft.cbrc.jp/alignment/server/index.html>), followed by shading with the Jalview 2.9.0b2 software (<http://www.jalview.org/>) (Waterhouse *et al.*, 2009). Generation of the phylogenetic tree was performed by the MEGA v6 software (Tamura *et al.*, 2013). Sequences were aligned using MUSCLE software. A maximum-likelihood tree was generated with a bootstrap value of 1000. Accession numbers for all sequences used are shown in Table S1.

Generation of the *cpsA* deletion and complementation strains

The DNA cassette used to delete *cpsA* was obtained by fusion PCR as previously described (Szewczyk *et al.*, 2006). The 1.4 kb 5' UTR and 1.5 kb 3' UTR flanking regions of *cpsA* were PCR amplified with RM1-F1 and cps-P2, and cps-P3 and RM1-R2A primers sets, respectively (Table S6). The *A. fumigatus pyrG* selectable marker was amplified with cps-P5 and cps-P6 primers from plasmid p1439 (Stinnett *et al.*, 2007). The 5' and 3' UTR fragments were then PCR fused to *pyrG^{Afum}* to generate the *cpsA* replacement construct using primers RM1-F2 and RM1-R4. RDAE206 and RJMP1.49 were used as host strains. The resulting transformation strains were designated as TDAED*cpsA* and TRVD*cpsA*, respectively. Genomic DNA from the selected transformants was analyzed by Southern blot.

A complementation strain was also obtained by transforming TRV *cpsA* with the *cpsA* wild-type allele. The complementation vector was generated as follows: a DNA fragment containing the entire *cpsA* wild-type locus, including 2.0 kb of upstream and 1.0 kb downstream regions, was amplified with the primers cps-com1 and cps-com2, digested with *EcoRI* and *HindIII* and ligated into pSM3 (containing *pyroA*), previously digested with the same enzymes, resulting in pSM3-*cpsA*-com. The complementation vector was then transformed into TRV *cpsA* using *pyroA* as selectable marker. Complementation was confirmed by PCR. The complemented strain was designated as TRV *cpsA*-com.

Generation of *cpsA* overexpression strain

To generate the *cpsA* overexpression strain, the entire *cpsA* coding region was first PCR amplified using the cps-OE1 and cps-OE2 primers, with engineered *AscI* and *NoI* sites (Table S6). The PCR product was then digested with *AscI* and *NoI*, and ligated into pTDS1 vector previously digested with the same enzymes. pTDS1 contains the *A. nidulans gpdA* promoter, *tpc* terminator and the *pyrG* marker. The resulting plasmid was denominated pTDS-cpsOE. The pTDS-cpsOE vector was transformed into RJMP1.49, and transformants were selected on appropriate selection medium without uridine and uracil, and confirmed by diagnostic PCR using *gpdA*promoF and cps-OE2 primers (Table S6).

Toxin analysis

Culture plates containing 25 ml of solid GMM were top-agar inoculated with 10⁶ spores/plate, and incubated at 37 °C in the dark. Three cores (16 mm diameter) were collected from each replicate plate and extracted with 5 ml of chloroform. Alternatively, strains were incubated in GMM liquid shaken cultures (10⁶ spores/ml) at 37 °C and 250 rpm. Culture

supernatants were collected and extracted with chloroform. Extracts were dried overnight and then re-suspended in 500 µl of chloroform. Samples were fractionated by thin-layer chromatography (TLC) using benzene and glacial acetic acid [95:5 (v/v)] as solvent system for ST analysis, and chloroform: acetone: n-hexane (85:15:20) for NOR analysis. Aluminum chloride (15% in ethanol) were then sprayed on the plates, and baked at 80 °C for 10 min. Metabolites present on TLC plates were visualized under 375 nm UV light. Commercial ST (Sigma) was used as standard.

PN analysis

The PN bioassay was performed as previously described (Brakhage *et al.*, 1992) with some modifications, using *Bacillus calidolactis* C953 as the test organism. Briefly, twenty-five milliliters of seed culture medium were inoculated using 10⁶ spores/ml of wild type, deletion *cpsA*, complementation and overexpression strains, and incubated at 26 °C for 48 h at 250 rpm. Mycelia were washed and transferred to 20 ml of fermentation medium. Mycelial samples were harvested at 24 h and 48 h for gene expression analysis. After 96 h, the culture supernatants were collected for PN analysis. Four-hundred milliliters of Tryptone-Soy Agar was supplemented with 30 ml of *B. calidolactis* C953 culture and plated on five 150-mm-diameter Petri dishes. Twenty microlitres of each culture supernatant were added to 7-mm-diameter wells. The plates were incubated at 55°C for 24 h, when inhibition halos were visualized and measured. To verify that the observed antibacterial activity was due to the presence of PN, and not to the presence of different fungal compounds in the supernatant, controls containing commercial penicillinase from *Bacillus cereus* (Sigma, MO, USA) were also included. A standard curve using various concentrations of PN G (Sigma, MO, USA) was utilized to determine PN concentration in the supernatants.

Morphological studies

Plates containing 25 ml of solid GMM medium were inoculated with 5 ml of top agar containing 10⁶ spores of wild type, *cpsA*, complementation and overexpression strains. The cultures were incubated in dark or light at 37°C. Cores (7 mm diameter top agar layer) were removed from each culture after 48 h and 72 h and homogenized in water. Conidia and Hülle cells were counted using a hemacytometer. Identical cores were also taken to visualize cleistothecia under a dissecting microscope. To improve visualization of fruiting bodies, the cores were sprayed with 70% ethanol to remove conidiophores. To examine colony growth, each strain was point-inoculated on GMM plates and incubated under light or dark conditions at 37 °C for 6 days. Colony diameter was measured. Experiments were performed with three replicates.

Gene expression analysis

Total RNA was extracted from lyophilized mycelia as previously described (Weber *et al.*, 1998). Gene expression levels were evaluated by Northern blot or quantitative reverse transcription-PCR (qRT-PCR) analysis. For Northern analysis, about 25 µg of total RNA of each sample was separated by agarose gel electrophoresis and blotted as described by Sambrook and Russell (2001). For qRT-PCR, total RNA was extracted from lyophilized mycelia using TRIsure (Bioline) reagent and RNeasy Mini Kit (Qiagen) according to the manufacturer instructions. Five µg of total RNA were treated with RQ1 RNase-Free DNase

(Promega). cDNA was synthesized with Moloney murine leukaemia virus (MMLV) reverse transcriptase (Promega). qRT-PCR was performed with the Applied Biosystems 7000 Real-Time PCR System using SYBR green dye for fluorescence detection. *A. nidulans* actin gene expression was used as reference, and the relative expression levels were calculated using the 2^{-CT} method (Livak and Schmittgen, 2001). Primer pairs used are shown in Table S6.

Study of *cpsA* subcellular localization

Aspergillus nidulans RJMP1.49 strain was transformed with a *cpsA::gfp::pyrG^{A.fum}* DNA cassette generated by fusion PCR as previously described (Szewczyk *et al.*, 2006). Specifically, the *cpsA* coding region and the 3' UTR were PCR amplified with RM1-F4 and *cpsgfp*-P2, and *cpsgfp*-P3 and RM1-R2A primers sets, respectively. The *gfp::pyrG^{A.fum}* fragment was amplified with *cpsgfp*-P5 and *cpsgfp*-P6 primers from plasmid p1439. The fragments were then PCR fused to generate the *cpsA::gfp::pyrG^{A.fum}* cassette using primers RM1-F5 and RM1-R3. Two transformants (TXF1.1 and TXF1.2) were selected for further study. An overexpression strain, *gpdA(p)::cpsA::gfp::pyrG^{A.fum}* was also constructed as follows: *cpsA::gfp* was PCR amplified from TXF1.1 genomic DNA using *cps*-OE1 and *GFP* + *NotI* primers (Table S6), digested with *AscI* and *NotI* and ligated to pTDS1 vector previously digested with the same enzymes. The resulting plasmid was designated as pSSP4.1. The vector was then transformed into TSSP7.1 resulting in strain TSS19.1. Conidia from the selected transformants, TXF1.1 TXF1.2 and TSS19.1 were inoculated as previously described (Stinnett *et al.*, 2007). Conidia were allowed to germinate on coverslip submerged in Watch minimal medium (WMM) (Peñalva, 2005) and incubated for 16 h. Time lapse microscopy was done using Stream acquisition with 200 ms exposure per frame using Metamorph software and a DMI6000b inverted microscope equipped with GFP and CMAC (UV) detection filters. Images were acquired with a Hamamatsu ORCA-ERII high-sensitivity monochrome digital CCD camera. Staining with CMAC (Molecular Probes) was carried out adding CMAC 10 μ M to the culture and incubating during 5 min at 25 °C followed by three washes with WMM. CMAC (7-amino-4-chloromethylcoumarin) is commonly used for vacuole staining (acidic pH in vacuoles activates this dye). Stack of images was converted to mov (Quicktime) using ImageJ software and QTmoviemaker plugging. Movie 3 (showing only motile particles) is a stack of images from Movie 2 subjected to "Walking average" subroutine of ImageJ (4 images for media calculation) and then to "stack difference" (image interval set to 1). Both ImageJ pluggings were obtained from EMBL, https://www.embl.de/eamnet/html/body_kymograph.html.

Protein extraction and Western blot

Conidia of wild type and *CpsA*-GFP strain were inoculated in GMM and cultivated at 37 °C for 18 h in an orbital shaker at 250 rpm. Mycelia were collected and lyophilized. Part of each mycelial mat was subjected to protein extraction with an alkaline lysis protocol and another part to standardized protein extraction using A50 buffer as indicated in Hernández-Ortiz and Espeso (2013). Only the soluble fraction of A50 buffer extraction is normally collected and used for further analyses. In this manuscript we kept the insoluble fraction, which was treated with the alkaline lysis protocol to analyze insoluble proteins or those linked to endomembranes or plasma membrane. SDS-PAGE and Western blotting was performed using standard procedures. Proteins were transferred to nitrocellulose using the

Transblot System from BioRad. Mouse antibodies clones 7.1 and 13.1 against GFP were obtained from Sigma (1/5000 dilution). Secondary anti-mouse antibody was obtained from Jackson (1/4000 dilution). Western blot was developed using the ECL chemiluminescent kit. Images were taken using a BioRad Chemidoc Imaging system and processed using Image Lab software (BioRad).

Cell wall stress assay

To test for possible alterations in the cell wall caused by either deletion or overexpression of *cpsA*, we exposed the wild type, *cpsA*, complementation and overexpression strains to SDS or calcofluor white (CFW) as previously described by Riche *et al* (2009). GMM was amended with various concentrations of SDS (0, 0.001%, 0.002%, 0.003%, 0.004%, 0.005%), or calcofluor white (CFW) (0, 2, 4, 6, 8, 10 µg/ml). The strains were then point-inoculated on these media, and incubated at 37 °C for 3 days.

Cell wall chemical analysis

Conidia (10^6 spores/ml) from wild type, *cpsA*, complementation and overexpression strains were inoculated in 50 ml of liquid GMM and incubated for 48 h at 37 °C and 250 rpm. Mycelium was harvested by filtration using miracloth, and washed twice with sterile distilled water. Five milligrams of dry mycelium was finely ground. Each sample was boiled in 1 ml of buffer A (2% SDS, 50 mM Tris-HCl, 100 mM Na-EDTA, 40 mM β-mercaptoethanol, 1 mM PMSF) for 15 min (Riche *et al.*, 2009). Mannoprotein was extracted with 1M NaOH at 75 °C for 1 h and quantified as previous described (de Groot *et al.*, 2008; Kapteyn *et al.*, 2001). Glucan and chitin were digested in 96% formic acid at 100 °C for 4 h (Li *et al.*, 2006). Formic acid was evaporated and residues were dissolved in 1 ml of distilled water. The contents of glucan and chitin were evaluated by the quantification of released glucose and N-acetylglucosamine after digestion. Glucose concentration was measured by the phenol-sulphuric acid method at 490 nm (Dubois *et al.*, 1956). N-acetylglucosamine concentration was measured at 520 nm by the method described by Lee *et al* (2005). The experiment included five replicates.

Transmission Electron Microscopy

Samples were fixed in 2.5% glutaraldehyde in Millonig's phosphate buffer for 3 h at 4 °C, rinsed (3×10 min) in Millonig's phosphate buffer followed by a treatment with 1% osmium tetroxide (OsO_4) for 2 h at room temperature. Then the samples were rinsed again (3×10 min) in Millonig's phosphate buffer, dehydrated in graduated ethanol series and cleared in acetone. The specimens were embedded using an EMBED-812 kit (Electron Microscopy Sciences) and sectioned with a Reichert Om-U2 ultramicrotome using a Diatome diamond knife. Samples were stained with 2% uranyl acetate (Watson) followed by lead citrate (Sato). The fixation/dehydration/embedding was repeated twice (two separate experiments) with similar results. For each samples, twenty images were visualized with a Hitachi H-600 transmission electron microscope. Micrographs were taken with a SIA L12C digital camera operated using MaxIm DL version 5.08 software.

Adhesion assay

The possible effect of *cpsA* on *A. nidulans* adhesion capacity was tested using 96 well plates as previously described (Mowat *et al.*, 2007; Pierce *et al.*, 2008; Coffey and Anderson, 2014). Briefly, one hundred and thirty microliters of inoculated GMM (10^5 spores/ml) were placed in each of the 96 wells on polystyrene culture plates, and incubated at 37 °C for 24 h, 48 h and 72 h respectively. Then, the supernatant was removed and the mycelium attached to the surface was washed 3 times with distilled water before adding 130 μ l of Crystal violet 0.01% in water. After 20 min at room temperature, the wells were washed 3 times with distilled water and were dried at room temperature. One hundred and thirty microliters of acetic acid 30% were used for distaining. OD was measured at 560 nm.

Oxidative and osmotic stress assays

In order to test whether *cpsA* affects *A. nidulans* response to oxidative stress, a method described by Tribus *et al.* (2005) was used with some modifications. Solid GMM containing various concentrations of menadione (0, 0.04, 0.08, 0.12, 0.16, 0.20 mM) in 24 well plates were point-inoculated with the wild type, *cpsA*, complementation and overexpression strains and incubated at 37 °C for 3 days. GMM without menadione was used as control.

Expression analysis of *thiO* and *catB* genes was examined by qRT-PCR and Northern blot, respectively, using the primers listed in Table S6. The strains (10^6 spores/ml) were inoculated in liquid GMM and incubated at 37°C in the dark for 48 h at 250 rpm. After that, approximately the same amount of mycelium from each strain was transferred to GMM with or without menadione (0.08 mM).

To test sensitivity to osmotic stress, the wild type, *cpsA*, complementation and overexpression strains were point-inoculated on GMM and GMM supplemented with 0.7 M NaCl, or 1.2 M sorbitol. The cultures were also grown at different temperatures, 30 °C, 37 °C and 45 °C, to examine whether *cpsA* function is affected by this parameter.

Generation of *rlmA* and *veA*, *rlmA* double mutant

The DNA cassette used to delete *rlmA* by gene replacement was obtained by the fusion PCR method as previously described (Szewczyk *et al.*, 2006). The 1.3 kb 5' UTR and 1.5 kb 3' UTR of *rlmA* were amplified from genomic DNA with primers ANrmlA_P1, ANrmlA_P2 and ANrmlA_P3, ANrmlA_P4, respectively. The *A. fumigatus pyrG* selectable marker was amplified with ANrmlA_P5_pyrGafum and ANrmlA_P6_pyrGafum primers from plasmid p1439. The 5' UTR and 3' UTR fragments were fused to *pyrG^{A.fum}* using ANrlm_p7 and ANrlm_p8 primers. TSSP7.1 strain was utilized as host for the transformation, generating TSSP15.1. The DNA cassette was also used to delete *rlmA* in TXF3.1 (*veA*), generating TSSP16.1. Deletion of *rlmA* and *veA* was confirmed by PCR.

Analysis of hyaluronic acid and glycosyl composition of cell wall fractions

Plates containing 25 ml of liquid GMM were inoculated with 10^6 spores/ml of wild type, *cpsA*, complementation and overexpression strains. The cultures were then incubated in dark at 37 °C for 48 h, when mycelium was collected by filtration using Miracloth. The cell wall samples were obtained as follows: mycelium was first washed with 1xPBS and

lyophilized. Then samples were ground, washed with 1xPBS four times, and boiled in water for 10 min, followed by four additional washes with 1xPBS and water. The cell wall samples were sent to the Complex Carbohydrate Research Center (Athens, GA) for analysis. Approximately 0.5 g of each cell wall fraction was suspended in 5 mL of HA buffer (50 mM sodium acetate, pH 6) and hyaluronase (from *Streptomyces hyalurolyticus*), final concentration 0.05 U/mL. The digestion was carried out at 55 °C for 24 h. A second aliquot of enzyme was added at that time and the reaction was allowed to continue for another 24 h. The reaction was then stopped by boiling for 10 min and filtered through a C18 SPE cartridge to separate out any remaining solids and to remove any remaining protein from solution. The filtrate was then lyophilized and reconstituted in 500 µl of water and analyzed by HPLC. Compounds were separated using an Agilent system with a 4.6×250 mm Waters Spherisorb analytical column with 5µm particle size at 25°C, and a solvent gradient (solvent A: 2.5 mM sodium phosphate, pH 3.5 and solvent B:) 2.5 mM sodium phosphate, 1.2 M NaCl, pH 3.5), and a flow rate of 1 mL/min. Detection was performed by post-column derivatization. Briefly, the eluent from the column was combined with a 1:1 mixture of 0.25 M NaOH and 1% 2-cyanoacetamide pumped at a flow rate of 0.5 mL/min from a post column reactor. The eluent was heated to 130 °C in a 10-m reaction coil, then cooled in a 50-cm cooling coil and measured with a fluorescence detection ($\lambda_{ex} = 346 \text{ nm}$, $\lambda_{em} = 410$). Identification and quantification was determined by comparison with a digestion of Hyaluronan as positive control.

Cell wall monosaccharide components were analyzed by GC-MS. Approximately 2 mg of wild type and *cpsA* cell wall samples were placed in screw-cap tubes, 1 µL of 1 mg/mL inositol was added as internal standard, and the samples were lyophilized. Methyl glycosides then were prepared from the dried samples by methanolysis with 3 M HCl in methanol at 100 °C for 2 h, and re-N-acetylation with pyridine and acetic anhydride in methanol. The samples then were per-O-trimethylsilylated (TMS) with a Tri-Sil reagent (Thermo Scientific) at 80 °C for 30 min. Analysis of the TMS methyl glycosides was performed on a Agilent 7890A gas chromatograph equipped with a Supelco EC-1 fused silica capillary column (30 m × 0.25 mm ID) and interfaced to a Agilent 5975C inert MSD.

Analysis of EPS

To assess whether hyaluronan is present in EPS of *A. nidulans*, its glycosyl composition of the EPS was analyzed by GC-MS in our laboratory. EPS was first precipitated from culture supernatants of wild type and *cpsA* mutant strain with 1 volume of ethanol 96% and dialyzed with water using a membrane with a 3 kDa cutoff. Samples were first hydrolyzed with 3 M trifluoroacetic acid (TFA) at 121 °C for 1 h, and derivatized and analyzed as previously reported (Bernabé *et al.*, 2011).

In addition, EPS was also analyzed by infrared spectroscopy. Infrared spectra were obtained by the KBr technique. In brief, approximately 2 mg of dry sample were thoroughly mixed in a mortar with 300 mg of KBr and maintained in a desiccator. The pellet was prepared by using a hydraulic press, applying a pressure of 2 tons for 2 minutes and then 10 tons for 6 min. The spectra were recorded in a FTIR 4200 type A instrument (Jasco Corporation,

Tokyo, Japan). Light source of transmittance was in the middle range infrared 700–4000 cm^{-1} . The detector used was triglycine-sulfate (TGS) with resolution 4 cm^{-1} .

The type of linkages in the EPS was also analyzed after methylation of dry samples (1–3 mg). Then samples were dissolved in dimethyl sulfoxide and processed according to the method of Ciucanu and Kerek (1984). The per-*O*-methylated polysaccharides were hydrolyzed with 3 M TFA, reduced with NaBD₄, derivatized to their corresponding partially methylated alditol acetates, and analyzed GC-MS as previously described (Notararigo et al., 2013).

Analysis of monosaccharides in membrane

Protoplasts from the wild-type strain TRV50.2 were prepared from mycelium grown on GMM for 16 h. Four grams of mycelium was treated with Glucanex (Novozymes) for 1.5 h in 1.2 M MgSO₄, 10 mM phosphate buffer at pH 6.5. Protoplasts were purified as described in the transformation protocol from Tilburn et al (1983) and lyophilized. The sample was hydrolyzed with TFA under the same conditions described for EPS analysis, and the compounds released were derivatized both as alditol acetates and as TMS-oximes before GC/MS analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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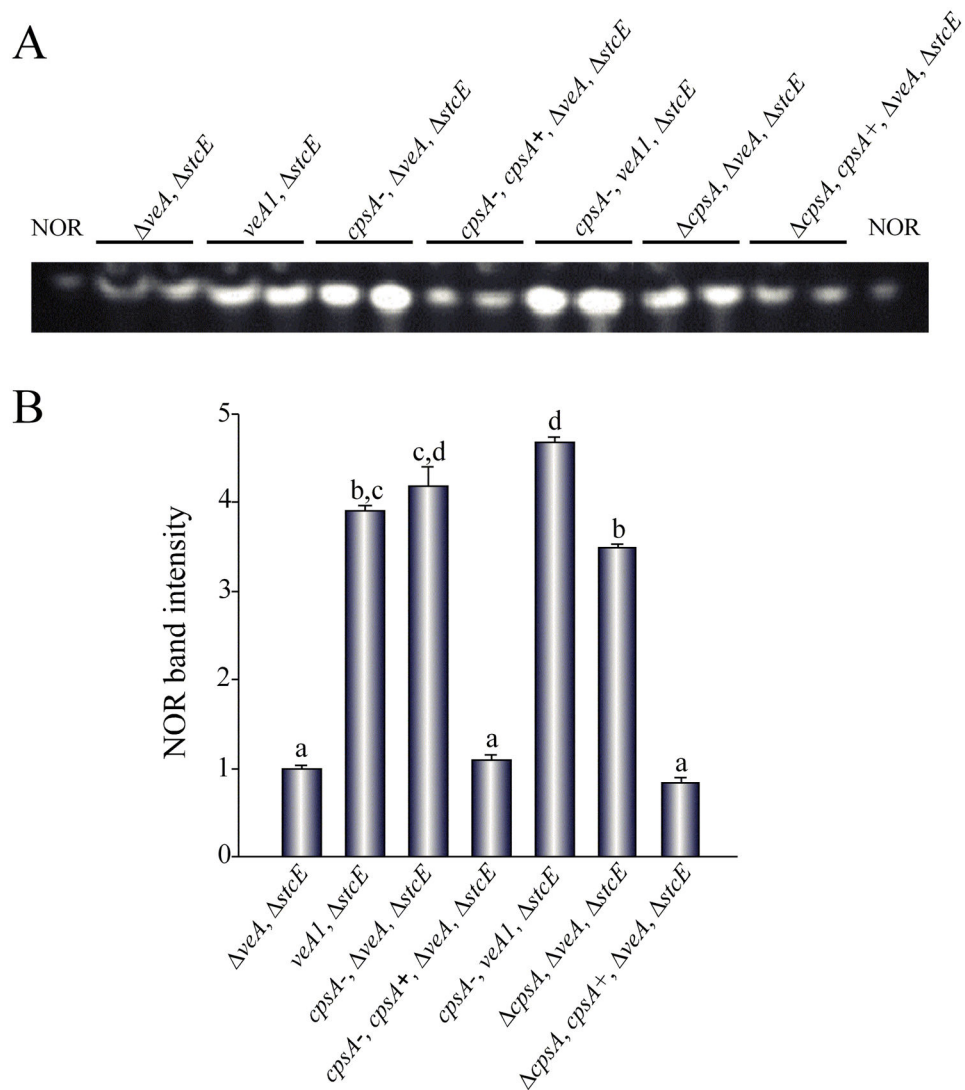


Figure 1. TLC analysis of NOR production in GMM cultures

(A) The strains *veA stcE* (RDAEp206), *veA1 stcE* (RAV1p), *cpsA- veA stcE* (RM1p), *cpsA- cpsA+ veA stcE* (RM1p-com), *cpsA- veA1 stcE* (RM1p-R2), *cpsA veA stcE* (TDAE *cpsA*), *cpsA cpsA+ veA stcE* (TDAE *cpsA-com*) (Table S2), were top-agar inoculated and incubated at 37 °C in the dark for 5 days. NOR production was analyzed by TLC. (B) Intensity of NOR bands. Densitometry was carried out with GelQuant.NET software. NOR band intensity was normalized using the intensity corresponding to RDAEp206 as 1. The letters above the bars represent significantly different values ($P < 0.05$, Tukey test).

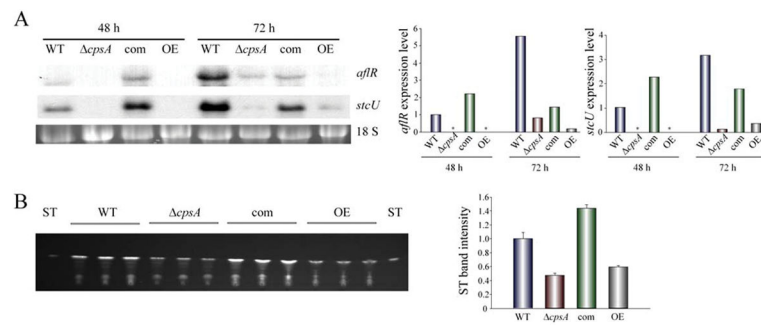


Figure 2. *cpsA* is necessary for normal expression of ST genes and mycotoxin production in *A. nidulans* strains with a *veA*⁺ wild-type background

(A) Wild type (TRV50.2) (WT) *veA*⁺ control, *cpsA* (TRVp *cpsA*), complementation (TRVp *cpsA*-com)(com), and overexpression (OE*cpsA*)(OE) strains were inoculated in liquid GMM. Mycelia were collected after 48 h and 72 h of culture in a shaker incubator at 250 rpm at 37 °C. Expression of *afIR* and *stcU* was analyzed by Northern blot. rRNA serves as loading control. Asterisk indicates not detected. Densitometry of the Northern blot results is shown. (B) TLC analysis of ST production in 72 h cultures described in (A), densitometry of ST band intensity is shown.

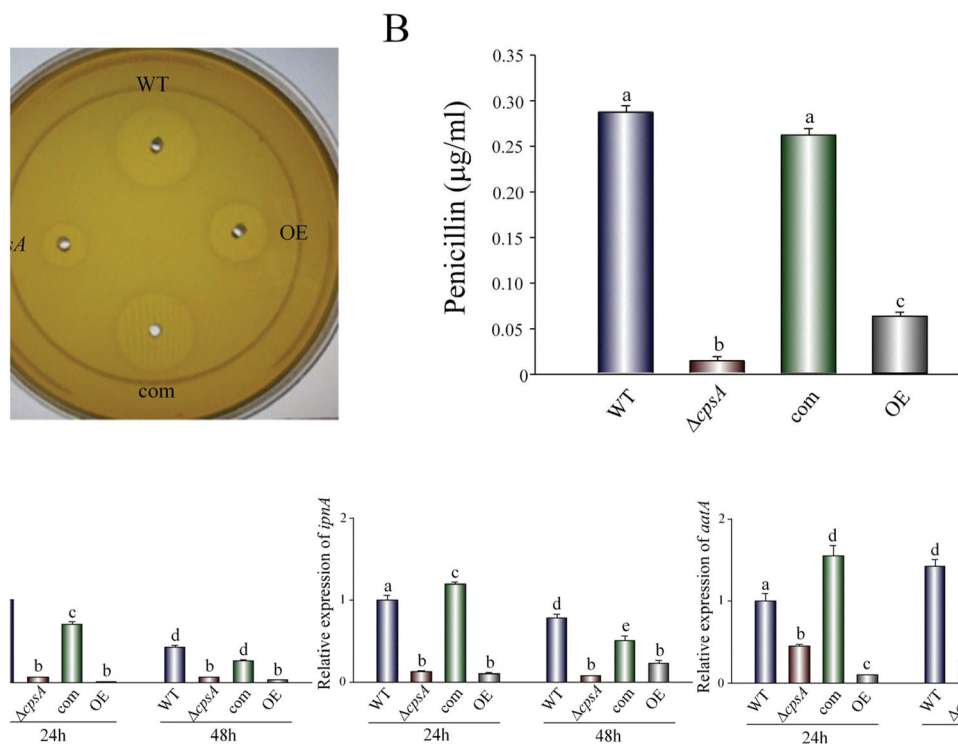


Figure 3. *cpsA* affects penicillin production

A) Extracts from wild-type (WT) *veA+* control, *cpsA*, complementation (com) and overexpression (OE) strains were analyzed for penicillin content as described in Materials and Methods section. (B) Quantification of penicillin content in the analyzed extracts. Values are means of three replicates. (C) qRT-PCR gene expression analysis by of *acvA*, *ipnA* and *aatA* from mycelial samples collected after 24 h and 48 h of incubation in PN inducing medium. The relative expression was calculated using the 2^{-CT} method, as described by Livak and Schmittgen (2001). Values were normalized to the expression levels in the wild type, considered 1. Error bars represent standard errors. The letters above the bars represent significantly different values ($P < 0.05$).

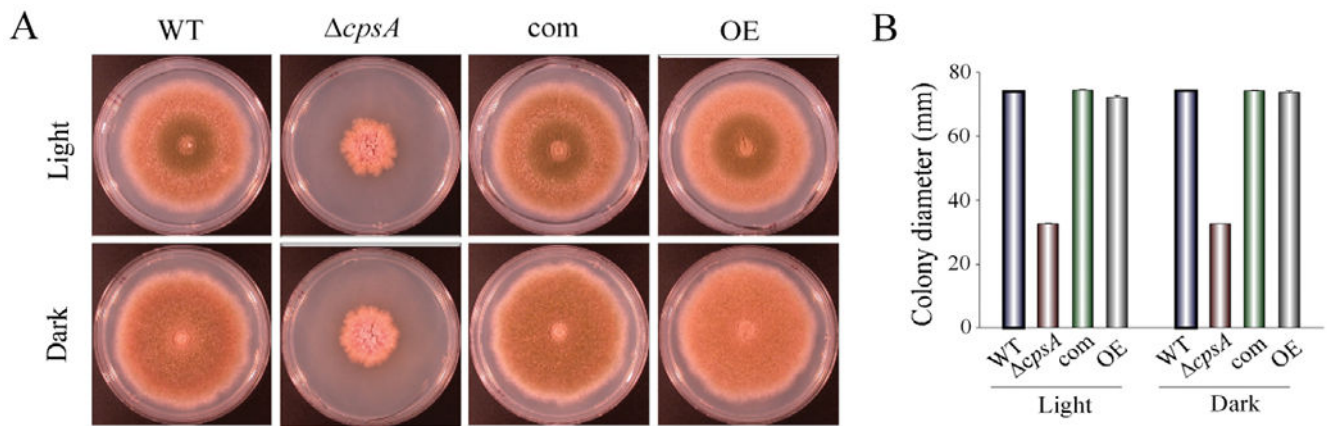


Figure 4. *cpsA* positively affects *A. nidulans* colony growth

(A) Photographs of wild-type (WT) *veA*⁺ control, *cpsA*, complementation (com) and overexpression (OE) strains point-inoculated on GMM plates and incubated at 37 °C in either light or dark for 6 days. (B) Colony growth measured as colony diameter. Standard error is shown. Means are average of three replicates.

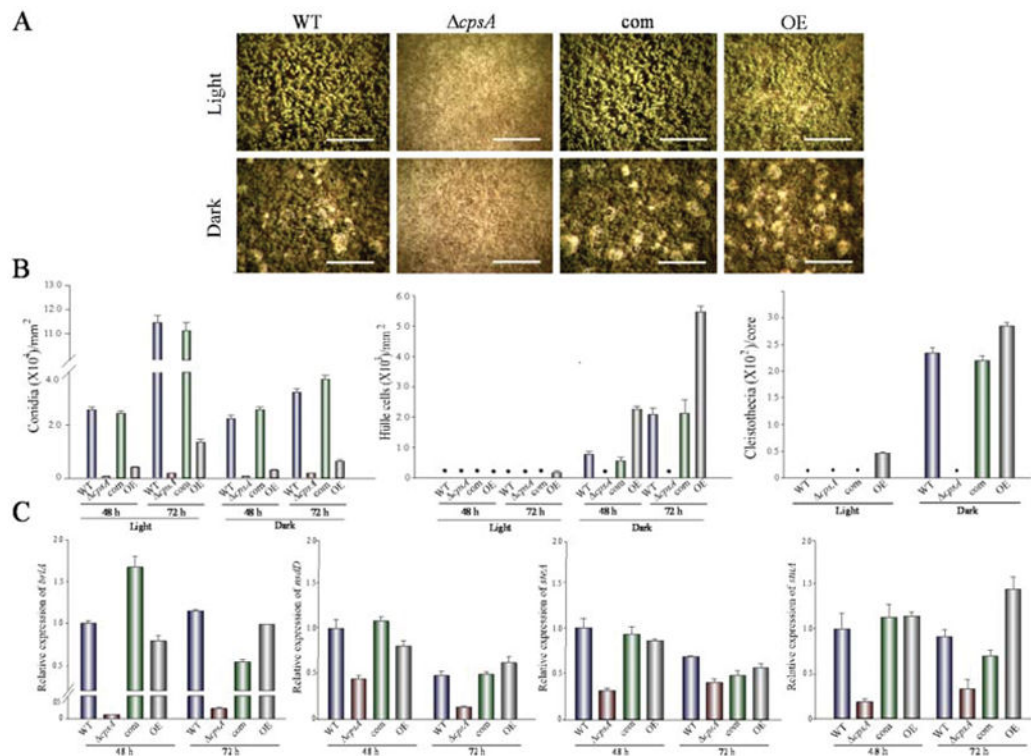


Figure 5. *cpsA* is necessary for proper asexual and sexual development

(A) Micrographs of wild-type (WT) *veA*⁺ control, *cpsA*, complementation (com) and overexpression (OE) strains point-inoculated GMM cultures incubated at 37 °C in either light or dark for 6 days. Samples were observed two centimeters from the inoculation point. Images were captured with an upright Leica MZ75 stereomicroscope. Scale bar corresponds to 200 μ m. (B) Quantitative analysis of production of conidia, Hülle cells and cleistothecia from top-agar inoculated GMM cultures incubated at 37 °C in either light or dark for 48 h and 72 h, in the case of conidia and Hülle cells, and 6 days in the case of cleistothecia. Asterisk indicates not detected. The experiment included three replicates. (C) qRT-PCR gene expression analysis of *brlA*, *nsdD*, *steA* and *stuA* from mycelial samples from 48 h and 72 h GMM liquid stationary cultures. The relative expression was calculated using the 2^{-CT} method, as described by Livak and Schmittgen (2001). Values were normalized to the expression levels in the wild type, considered 1. Error bars represent standard errors.

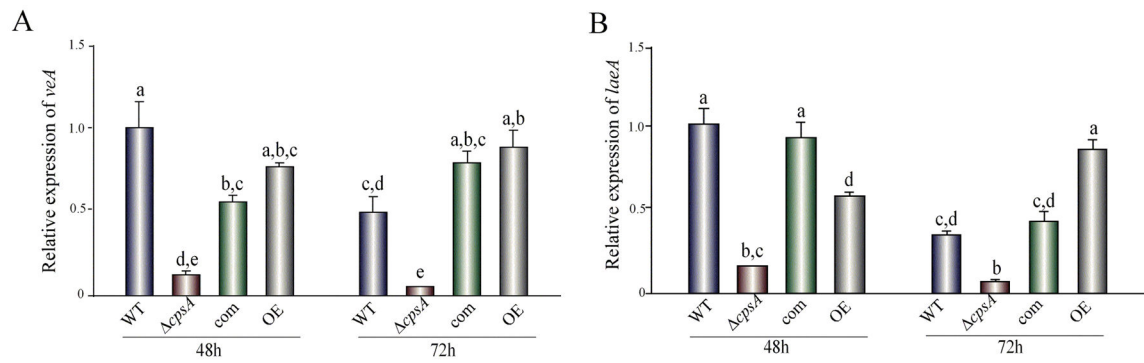


Figure 6. Expression of *veA* and *laeA* is positively affected by *cpsA*

qRT-PCR gene expression analysis of *veA* and *laeA* from liquid stationary GMM cultures 48 h and 72 h after incubation in the dark at 37 °C. The relative expression was calculated using the 2^{-CT} method, as described by Livak and Schmittgen (2001). Levels in the wild type, considered 1. Error bars represent standard errors. The letters above the bars represent significantly different values ($P < 0.05$).

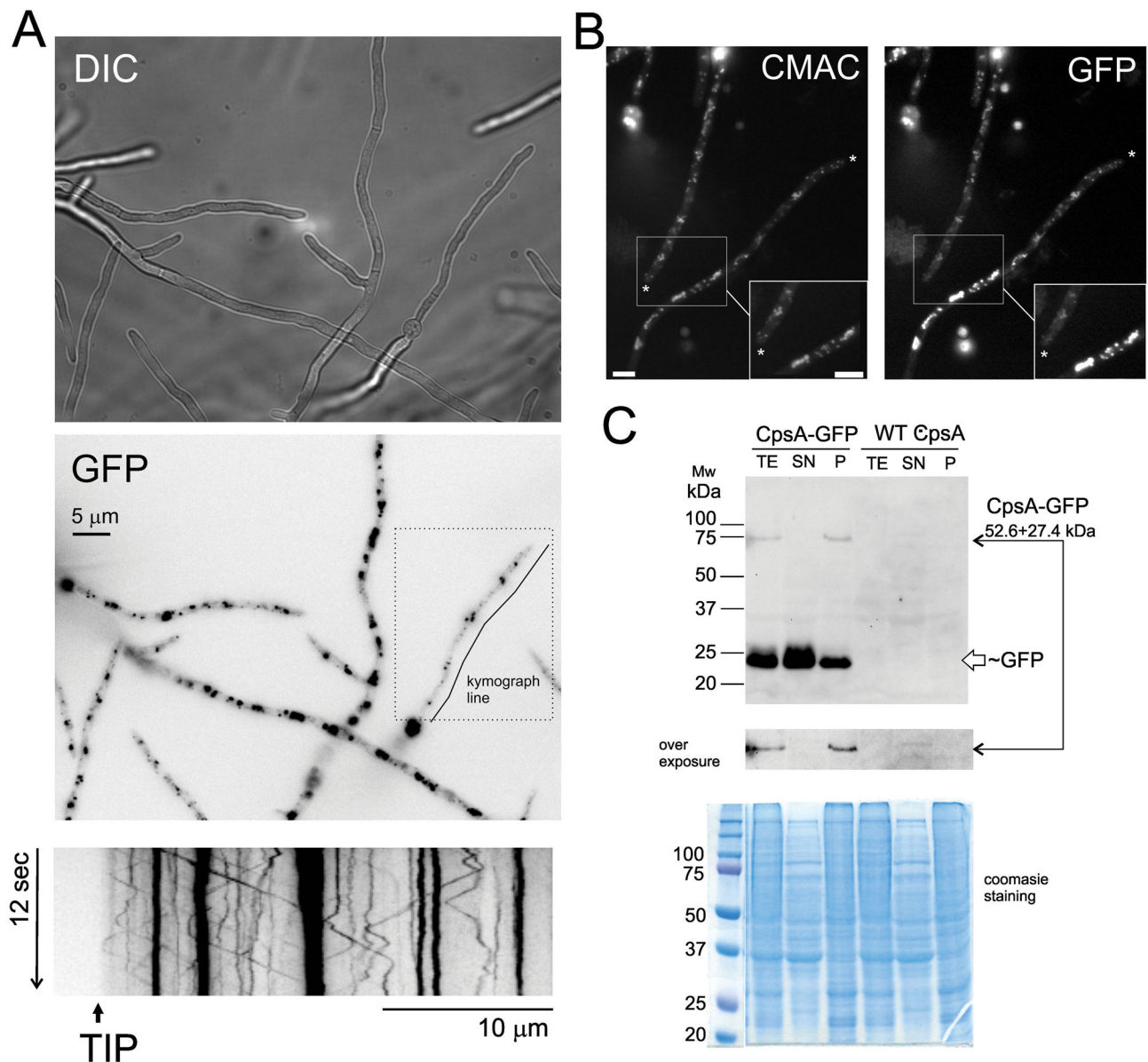


Figure 7. Localization of CpsA protein

(A) Cellular localization of CpsA-GFP fusion protein expressed by its endogenous promoter. Cells were grown for 16 h at 25 °C. CpsA accumulates at small vesicles resembling vacuoles and endosomes. The kymograph obtained from a stack of 90 images for 12 seconds (200 ms exposure each) along the line shown in GFP inset. Dashed line square indicates the limits of Movie 1. Kymograph shows the presence of static and motile vesicles migrating in an anterograde (to the tip) and retrograde (from tip to base of cells) modes. (B) CMAC staining confirm the presence of CpsA-GFP in small vacuoles. Details of the aggregates are shown at the bottom of the images. Asterisks indicate cell tips. Scale bar represents 5 micrometers. (C) Detection of CpsA-GFP fusion using GFP antibodies. TE indicates a total protein extract using an alkaline lysis protocol. Soluble protein from A50 extraction protocol is shown in line SN. Insoluble protein in A50 treatment was analyzed in line P. An overexposure of the

filter on the CpsA-GFP fusion region is shown. In both protocols a band corresponding to GFP is visualized as indication of protein degradation in vacuoles or MVs (multivesicular bodies)/lysosomes.

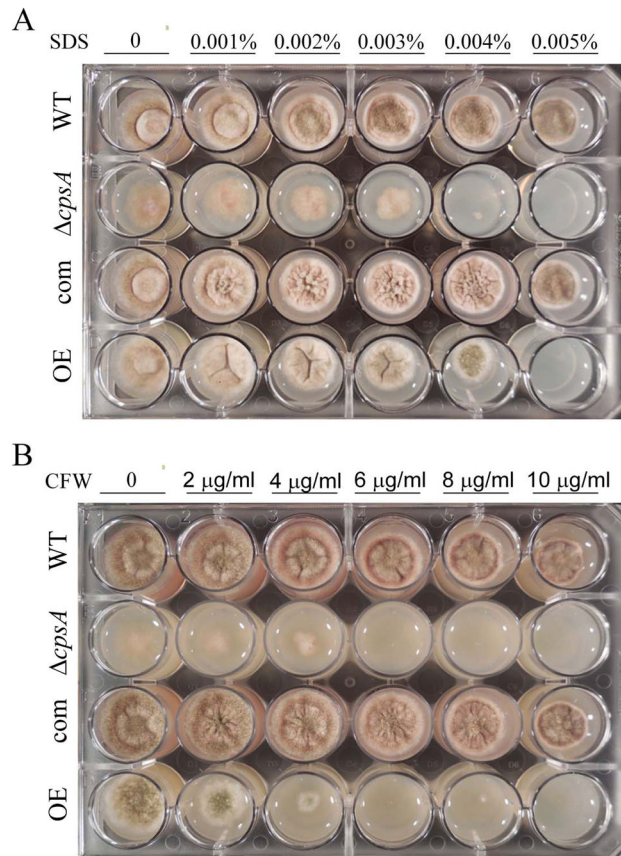


Figure 8. Absence of or overexpression of *cpsA* increases sensitivity to SDS and CFW
 (A) Wild-type (WT) *veA*⁺ control, *cpsA*, complementation (com) and overexpression (OE) strains were point inoculated on GMM containing SDS (A) and CFW (B) at different concentrations. Cultures were incubated at 37 °C in the dark for 3 days.

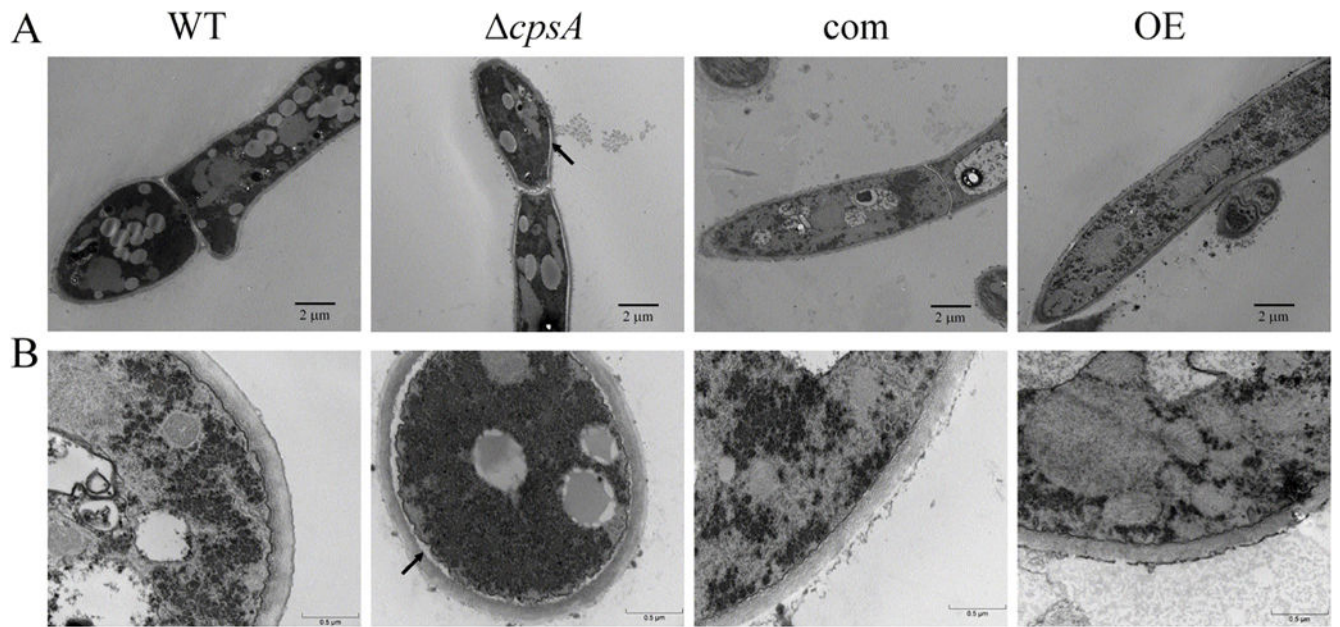


Figure 9. *cpsA* is required for proper attachment of the cell wall to the plasma membrane
 Transmission electron microscopy images of wild-type (WT) *veA*⁺ control, *cpsA*, complementation (com) and overexpression (OE) strains. The strains were grown in liquid stationary GMM cultures at 37 °C for 48 h. Mycelia were fixed and stained with 2% uranyl acetate and 0.2% lead citrate. The arrows show the cell structure alteration with detachment of plasma membrane and cell wall. Micrographs were acquired with different magnifications represented by panels in (A) and (B).

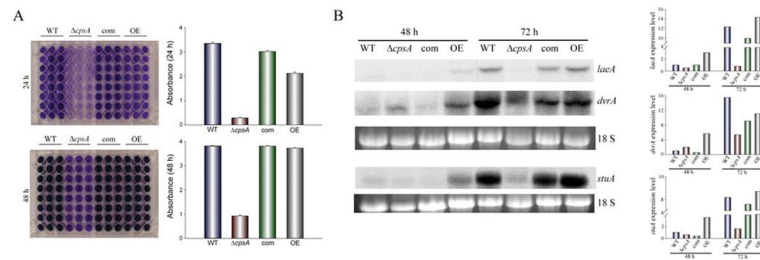


Figure 10. *cpsA* is involved in adhesion to surfaces

(A) Conidia of wild-type (WT) *veA*⁺ control, *cpsA*, complementation (com) and overexpression (OE) strains were inoculated in liquid GMM (10^5 spores/ml), and incubated at 37 °C for 24 h and 48 h as stationary cultures. Mycelium was stained with Crystal violet as described in Materials and Methods section. The 48 h samples in panel A were diluted 2-fold for measurement at 560 nm. (B) Expression analysis of *laeA*, *dvrA*, and *stuA* by Northern blot. The strains were inoculated (10^5 spores/ml) in Petri dishes containing 20 ml of liquid GMM, and incubated at 37 °C for 48 h and 72 h as stationary cultures. rRNA serves as loading control. Densitometry of the Northern blot results is shown.

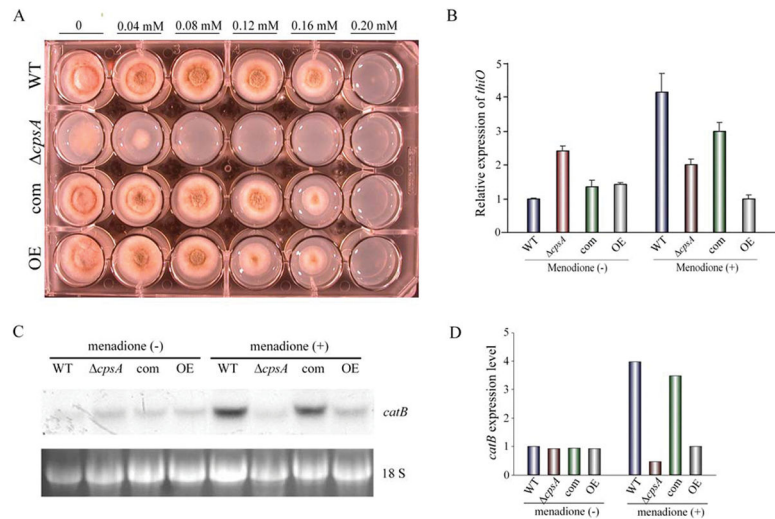


Figure 11. *cpsA* is necessary in resistance to oxidative stress in *A. nidulans*

(A) Wild-type (WT) *veA*⁺ control, *cpsA*, complementation (com) and overexpression (OE) strains were point-inoculated on GMM containing different concentrations of menadione and incubated at 37 °C in dark for 3 days. Expression analysis of *thiO* by qRT-PCR (B) and *catB* by Northern blot (C and D). The strains were incubated in liquid GMM at 250 rpm at 37°C in the dark for 48 h. After that, mycelia were transferred to GMM with or without menadione (0.08 mM). rRNA serves as loading control. Densitometry of the Northern blot results is shown.

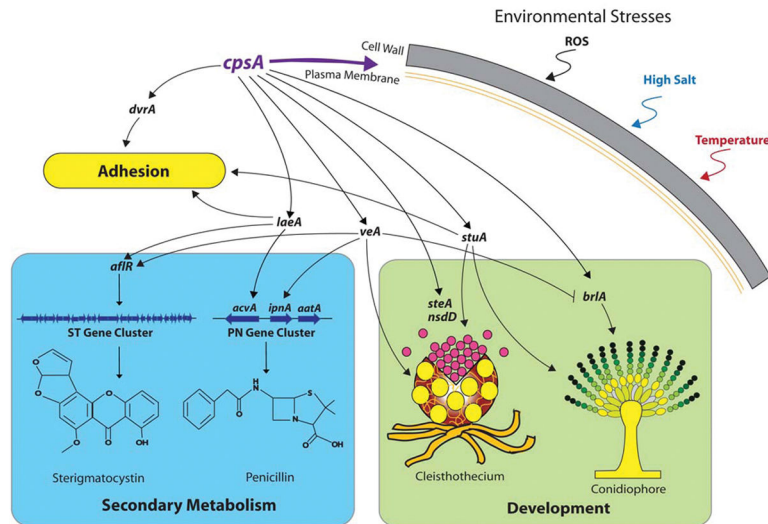


Figure 12. Model of the role of *cpsA* in *A. nidulans*

cpsA is necessary for proper attachment of the cell wall to the plasma membrane, and the normal cell wall composition and integrity that protect fungal cells from environmental stresses. All the genes in this model are downregulated in the absence of *cpsA*. The *cpsA* gene positively influences the expression of the master regulators *veA*, *laeA* and *stuA*, and is required for normal sexual and asexual development, secondary metabolism and adhesion capacity.

Table 1

Composition of cell wall ($\mu\text{g}/\text{mg}$ dry weight \pm standard deviation).

	Alkali-Soluble		Alkali-Insoluble	
	mannoprotein	glucan	glucan	chitin
WT	38.8 \pm 4.2 A	104.3 \pm 4.3 A	129.0 \pm 15.2 A	100.2 \pm 7.2 A
<i>cpsA</i>	17.7 \pm 4.3 C	83.6 \pm 6.8 B	84.8 \pm 7.9 B	80.0 \pm 5.3 B
com	39.6 \pm 7.5 A	112.8 \pm 9.1 A	137.2 \pm 15.0 A	107.9 \pm 5.2 A
OE	24.8 \pm 5.1 B	95.1 \pm 9.3 AB	80.3 \pm 5.6 B	82.0 \pm 7.0 B

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