Chapter 5

Molecular genetic analysis of vesicular transport in *Aspergillus niger* reveals partial conservation of the molecular mechanism of exocytosis in fungi

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Microbiology 2014 Feb;160(Pt 2):316-329

Abstract

The filamentous fungus *Aspergillus niger* is an industrially exploited protein expression platform, well known for its capacity to secrete high levels of proteins. To study the process of protein secretion in *A. niger*, we established a GFP-v-SNARE reporter strain in which the trafficking and dynamics of secretory vesicles can be followed *in vivo*. The biological role of putative *A. niger* orthologs of seven secretion-specific genes, known to function in key aspects of the protein secretion machinery in *S. cerevisiae*, was analyzed by constructing respective gene deletion mutants in the GFP-v-SNARE reporter strain. Comparison of the deletion phenotype of conserved proteins functioning in the secretory pathway revealed common features but also interesting differences between *S. cerevisiae* and *A. niger*. Deletion of the *S. cerevisiae* Sec2p ortholog in *A. niger* (SecB), encoding a guanine exchange factor for the GTPase Sec4p (SrgA in *A. niger*), did not have an obvious phenotype, while *SEC2* deletion in baker's yeast is lethal. Similarly, deletion of the *A. niger* ortholog of the *S. cerevisiae* exocyst subunit Sec3p (SecC) did not result in a lethal phenotype as in *S. cerevisiae*, although severe growth reduction of *A. niger* was observed. Deletion of *secA*, *secH* and *ssoA* (the *A. niger* orthologs of *S. cerevisiae* Sec1p, Sec8p and Sso1/2p, respectively) showed that these genes are essential for *A. niger*, similarly to the situation in *S. cerevisiae.* These data demonstrate that the orchestration of exocyst-mediated vesicle transport is only partially conserved in *S. cerevisiae* and *A. niger.*

Introduction

As a member of the black aspergilli, *Aspergillus niger* is an important industrial microorganism. It is used for the production of various food ingredients, pharmaceuticals and industrial enzymes (Fleissner and Dersch 2010; Meyer et al. 2008; Meyer et al. 2011b). Its high protein secretion capacity together with high production of organic acids, like citric acid has stimulated the development of both genetic and genomic tools for *A. niger* to get insights into the molecular basis of these special properties. (Carvalho et al. 2010; Fleissner and Dersch 2010; Jacobs et al. 2009; Meyer et al. 2007a; Meyer et al. 2008; Meyer et al. 2010b; Meyer et al. 2011b; Pel et al. 2007). Using these tools, also more complex processes such as the protein secretion process can now systematically be studied (Carvalho et al., 2011, 2012, Kwon et al., 2012).

A. niger is well known for its outstanding capacity to secrete proteins into the growth medium. However, the number of genes predicted to function in protein secretion in *Aspergilli* (including *A. niger* and *A. nidulans*) or *S. cerevisiae* does not explain differences

among the secretion capacities of these species (Pel et al. 2007). Up to now, the mechanisms to explain the difference in secretion efficiency, which might include higher levels of secretory vesicles, more efficient packing of cargo load in vesicles or faster trafficking through the secretory pathway, are not known. Growth and secretion are considered to be tightly connected processes. Experiments in *A. niger* in chemostat cultures grown at identical growth rates on different carbon sources (xylose or maltose) revealed different protein production rates. The specific production rate of extracellular proteins on maltose was about three times higher compared to xylose at identical growth rates (Jørgensen et al., 2009). One possible mechanism to explain this uncoupling of growth and secretion in *A. niger* can be the existence of two parallel secretory pathways that independently deliver proteins destined for secretion (e.g. glucoamylase) and proteins destined for growth (e.g. plasma membrane proteins and cell wall synthesizing enzymes) to the cell surface. Several studies including studies in yeasts, plants and mammalian cells show that different populations of Golgi derived vesicles exist (Harsay and Bretscher 1995; Leucci et al. 2007; Titorenko et al. 1997; Yoshimori et al. 1996). Also in filamentous fungi, a study using *Trichoderma reesei* revealed the possible presence of more than one pathway for exocytosis based on spatial segregation of different SNARE complexes in the fungal tip cell (Valkonen et al. 2007).

The secretion process involves an ordered transport of proteins via various organelles which is mediated via secretory vesicles trafficking from one compartment to the next. The different transport steps along the secretory pathway involved in vesicle trafficking are mediated by the action of secretion-related small GTPases of the Ypt/Rab family (Segev 2001a). *A. niger* contains 11 different secretion-related GTPases that are expected to be involved in specific transport steps in the secretory pathway (Pel et al. 2007; Segev 2001a)). One of those, SrgA, the ortholog of Sec4p was described earlier to be involved in protein secretion but not being essential for the viability of *A. niger* (Punt et al. 2001). Another secretion related GTPase, SrgC, an ortholog of Rab6/Ypt6 was recently described to be required for maintaining the integrity of Golgi equivalents in *A. niger* (Carvalho et al. 2011b).

Other important factors involved in the secretion pathway as mediators of vesicle docking and fusion with the membrane are soluble NSF (N-ethylmaleimide sensitive factor) attachment protein receptors (SNAREs) (Bonifacino and Glick 2004; Chen and Scheller 2001). Like the Ypt/Rab proteins, these proteins are highly conserved in eukaryotic cells and most SNAREs are C-terminally anchored transmembrane (TM) proteins present on vesicles (v-SNAREs) and target (t-SNAREs) membranes (Bonifacino and Glick 2004; Chen and Scheller 2001; Gupta and Brent Heath 2002). SNAREs are categorized into two classes based on whether they contain an arginine (R) or glutamine (Q) residue in their

SNARE central domain. Q-SNARE are further subclassified into Qa, Qb or Qc- types (Bock et al. 2001). Monomeric R-SNARE (v-SNARE) on the vesicle membrane and oligomeric Q-SNAREs on the target membrane form a stable four helices complex called as the SNARE complex at each fusion site (Bonifacino and Glick 2004). The localization of SNARE proteins have been systematically analysed in *A. oryzae* and supports the localized distribution of specific SNARE proteins at specific membranes (Kuratsu et al., 2007). In filamentous fungi, the localization of v-SNARE Snc1 and t-SNAREs Sso1 and Sso2 have been studied in detail in *T. reesei* (Valkonen et al., 2007). This SNARE-complex plays an important role in the fusion of Golgi-derived vesicles with the plasma membrane. The vesicle fusion event to the plasma membrane is promoted by the exocyst complex which provides the spatio-temporal information for the initial recruitment and tethering of Golgi-derived secretory vesicles to the plasma membrane. The exocyst is a conserved eukaryotic multi-subunit complex composed of eight protein members: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84. It is localized to limited regions of the plasma membrane by the interaction of Exo70p and Sec3p to Rho-GTPases and phosphatidylinositol 4,5,-bisphosphate (PIP2) (see for a recent review (Heider and Munson 2012)).

The availability of temperature sensitive (ts) secretion mutants in *S. cerevisiae* has formed a strong basis for understanding and identification of secretion pathway genes including the Sec components of the exocyst complex (Novick et al. 1980; Schekman 2010). However, tools and strategies for selecting secretion mutants in filamentous fungi are lacking so far which is one reason why little is known about the regulation of the secretory pathway in filamentous fungi. In this study, we constructed an *A. niger* reporter strain expressing GFP-tagged v-SNARE to visualize secretory vesicle and used this strain to explore the function of seven predicted *A. niger* genes, which are homologous to *S. cerevisiae* genes playing a key role in the secretory pathway. The data obtained show that some genes are essential in both organisms, but also indicate interesting differences. The finding that some genes were not essential in *A. niger* but in *S. cerevisiae* indicates differences in the molecular mechanisms underlying the protein secretion process. For the essential *ssoA* gene, several approaches were undertaken to create a conditional secretion mutant of *A. niger*. Whereas attempts to introduce conserved temperature-sensitive mutations of the *S. cerevisiae* Sso1/Sso2p in the *A. niger* SsoA ortholog failed, a conditional *ssoA* mutant was obtained by controlled expression of *ssoA* in a *ssoA* deletion strain. Such a strain will facilitate synthetic lethal screens and the identification of high-copy number suppressors in future secretion-related studies.

Results

Localization of secretory vesicles in *A. niger*

The polarized delivery of secretory vesicles to the hyphal tips involves SNARE proteins as mediators of vesicles docking and fusion with the plasma membrane. SNARE proteins are organelle-specific thereby ensuring the fusion of a vesicle to the correct target membrane (Chen and Scheller 2001). In *S. cerevisiae* a redundant pair of highly homologous vesicular-SNARE (v-SNARE) proteins, Snc1p and Snc2p are required for the fusion of Golgi derived secretory vesicles with the plasma membrane (Protopopov et al. 1993). In order to examine the localization of secretory vesicles in *A. niger*, we constructed a reporter strain expressing a fusion protein of GFP and the v-SNARE protein, the homolog of the *S. cerevisiae* Snc1p/Snc2p proteins, named SncA in *A. niger* (Sagt et al. 2009). To minimize risks of non-functional protein expression and to prevent possible interference arising from non-physiological expression levels of the GFP-SncA fusion protein, GFP was fused to the open reading frame (ORF) of *sncA* at the N-terminus under control of its endogenous *sncA* promoter and used to replace the endogenous *sncA* gene. Notably, N-terminal tagging of SNAREs is favored over C-terminal tagging, as the C-terminal transmembrane (TM) domain is required for proper localization and function of SNAREs (Taheri-Talesh et al. 2008; Ungar and Hughson 2003). The expression cassette that targeted the fusion gene to the genomic locus of *sncA* in *A. niger* was constructed as depicted in Fig. 1a. After transformation, selected transformants were analyzed by Southern analysis and strain FG7 was selected as it contained the correct gene replacement (data not shown). FG7 was phenotypically indistinguishable from the wild-type strain with respect to growth at different temperatures as well as germination (data not shown).

The reporter strain FG7 was further analyzed by fluorescence microscopy. Bright GFP-SncA signals were observed along the hyphae but were more pronounced at the hyphal tips (Fig. 1b). The highest intensity of fluorescence was visible at the very apex of growing hyphae and at newly formed branches reminiscent of the Spitzenkörper, a vesicle-rich region present at actively growing hyphal tips of filamentous fungi also known as the vesicle supply center (Fig. 1b) (Harris et al. 2005; Steinberg 2007). The dynamic movement of vesicles in growing *A. niger* cells and the movement of the Spitzenkörper along the hyphal tip during growth were observed from four-dimensional image sets (Z-series captured over time, Supplemental video1) similar as described for *A. nidulans* and *A. oryzae* (Taheri-Talesh et al. 2008). To examine the role of the tubulin and actin cytoskeleton on the localization of secretory vesicles, the GFP-SncA reporter strain was treated with benomyl and latrunculin B, respectively, known to disrupt the

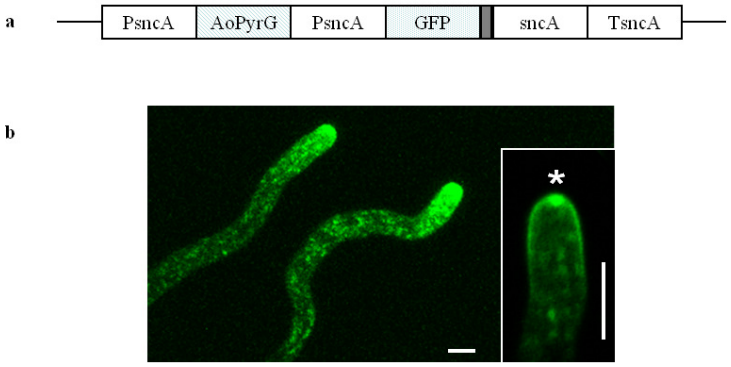

Fig. 1. Localization of v-SNARE protein SncA in living cells of *A. niger*. a) Schematic representation of the approach to label v-SNARE protein SncA with GFP. The GFP part was fused to the N-terminus of SncA and separated from SncA by a NPAFLYKVG-linker derived from the Gateway cloning technology. The construct is designed for integration at the *sncA* locus using the *A. oryzae pyrG* (*pyrG*) as a selection marker. The promoter region in front of the GFP-SncA fusion protein is about 800 bp to allow proper transcription and no interference of the *pyrG* gene. b) Confocal images of SncA localization in tip cells expressing GFP-SncA. The image represents a Z-stack of the entire hyphae showing the intracellular staining (representing secretory vesicles) as well as labeling of the plasma membrane. A clear gradient of GFP-SncA labeling towards the tip is visible. The Spitzenkörper is indicated with a star. Bar, 5 µm.

integrity and function of the cytoskeleton (Roca et al. 2010). As a control, GFP-tubulin (Kwon et al., 2011) and SlaB-YFP (Kwon et al., 2013) reporter strains were treated with the same concentration of benomyl and latrunculin B to confirm disruption of both the tubulin and actin networks by the concentrations used (Supplemental Fig. 2). As shown in Fig. 2, benomyl treatment of the GFP-SncA strain resulted in wider and curled hyphae and reduced the polar distribution of secretory vesicles at hyphal tips. Similarly, polar distribution of secretory vesicles was also lost when the function of the actin cytoskeleton was impaired by latrunculin B. Here, lower fluorescence and reduced polar accumulation of secretory vesicles at the hyphal tip was observed. These data demonstrate that the tubulin and actin cytoskeletal networks are crucial for targeted transport of secretory vesicles towards hyphal tips of *A. niger*.

Deletion of secretion-related genes in the GFP-SncA reporter strain

To identify proteins important for the delivery of vesicles to the plasma membrane, seven candidate genes covering different aspects of polarized protein secretion in *S. cerevisiae* including SNARE proteins, secretion related GTPase and members of the exocyst complex

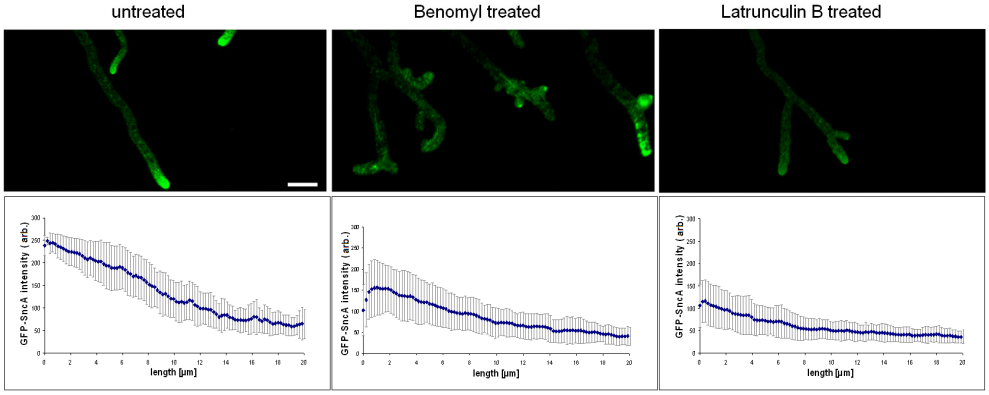

Fig. 2. Localization of v-SNARE protein SncA in living cells of *A. niger* after treatment with cytoskeleton disturbing compounds. Top panels: CLSM images showing the localization of GFP-SncA in hyphal tips. Left: untreated hyphae, middle: benomyl treated cells, right: latrunculin B treated cells. Lower panels: fluorescent intensity distributions along hyphal tip compartments (n > 20) within a region of 20 µm from the tip. Conidia of GFP-SncA strain were pre-grown on a MM agar plate for 2 days at 22°C and placed on a cover glass bottom culture dish containing MM media with 5 µg/ml of benomyl or 2 µg/ml of latrunculin B. After an additional hour incubation, the cells were examined using an inverted confocal microscope. Bar, 10 µm.

were selected (Table 1). These proteins were all selected based on high amino-acid sequence similarities with *S. cerevisiae* homologs (Pel et al., 2007, Table S2). Expression analysis of the different genes confirmed that all chosen genes are actively expressed during germination and exponential growth (Table 1). To study the roles of the seven genes and their effects on the localization of secretory vesicles in *A. niger*, respective deletion mutants were generated in both wild-type and GFP-SncA background strains. We were able to obtain viable deletion mutants for *secB*, *secC*, *srgA* and *sncA*; however, deletion of *secA*, *secH* or *ssoA* caused a lethal phenotype both in the wild-type as well as in the GFP-SncA background. Primary transformants for *secA*, *secH* or *ssoA* survived only as heterokaryons containing transformed (*ΔsecA/pyrG*$^{+}$) and untransformed nuclei (*secA/pyrG*$^{-}$) in the absence of uridine in the medium (data not shown). Correct deletion of the target genes in purified transformants (non-essential genes) or heterokaryons (essential genes) was verified by Southern analysis (Supplemental Fig. 1 and data not shown).

The growth phenotype as well as GFP-SncA localization for the viable deletion mutants was analyzed using plate growth assays and *in vivo* fluorescence microscopy. As shown in Fig. 3, deletion of the GTPase SrgA strongly reduced the growth rate of *A. niger* and resulted in the formation of a compact colony as previously reported (Punt et al. 2001). However, the localization of GFP-SncA in young germlings was not dramatically perturbed

Table 1. Expression and predicted function of selected secretion related genes in *A. niger*.

A. niger	*S. cerevisiae*	ORF code	Exponential growth phase[a]	Germination[b]	Predicted function
secA	*SEC1*	An14g03790	0.48 ± 0.02	0.48 ± 0.03	SNARE binding protein
secB	*SEC2*	An11g09910	0.74 ± 0.04	1.22 ± 0.30	Guanine exchange factor of SrgA
secC	*SEC3*	An01g03190	0.37 ± 0.01	0.43 ± 0.02	Subunit of the exocyst complex
srgA	*SEC4*	An14g00010	5.40 ± 0.23	1.97 ± 0.26	Rab GTPase
secH	*SEC8*	An03g04210	0.69 ± 0.03	0.57 ± 0.02	Subunit of the exocyst complex
ssoA	*SSO1/2**	An12g01190	1.14 ± 0.14	1.71 ± 0.04	t-SNARE
sncA	*SNC1/2**	An12g07570	5.18 ± 0.19	4.00 ± 0.03	v-SNARE

Mean expression values are given in % compared to the expression level of the actin gene *actA*. Data are taken from three independent cultivations: **a**: (Jørgensen et al. 2010), **b**: (Meyer et al. 2007b). * Sso1p and Sso2p as well as Snc1p and Scn2p are paralogs and have a redundant function. Deletion of both genes is lethal in *S. cerevisiae* (Protopopov et al., 1993; Jannti et al., 2002).

in the *ΔsrgA* strain despite the strong reduction in radial growth (Fig. 3). GFP-SncA localization was generally more intense along the hyphae but the majority of the signal resembled wild-type localization of secretory vesicles. SecB is the predicted guanine exchange factor (GEF) functioning as an activator of the GTPase SrgA. Interestingly, deletion of *secB* only mildly perturbed growth and did not resemble the expected Δ*srgA* phenotype. We thus examined whether another *sec2* homolog is present in the genome of *A. niger*. We noticed the presence of an uncharacterized protein of 257 amino acids in the *A. niger* genome (An15g06770) which contains a GDP/GTP exchange factor Sec2p domain (pfam06428) (Table S2). It will be of interest to determine whether this hypothetical protein which has orthologs in other filamentous fungi has an overlapping role with SecB. In agreement with the mild phenotype of the *ΔsecB* strain, the localization of GFP-SncA in the *ΔsecB* strain did not differ from the wild-type localization (Fig. 3).

The deletion of v-SNARE encoding gene *sncA* displayed a mild but significant phenotype with reduced radial growth (72%) when compared to the wild-type strain (Fig. 3). Morphologically, *ΔsncA* strain was identical to the wild-type strain. This was somewhat surprising since only a single copy of the *sncA* gene was found in the genome of *A. niger* ((Pel et al. 2007) and Table S2)).

In the case of *ΔsecC*, predicted to encode a subunit of the exocyst complex, the observed growth defect phenotype was very severe and characterized by strongly reduced growth and aberrant morphologies of young germlings (Fig. 4a). The *ΔsecC* strain in the wild-type background was able to grow on secondary selection plates only as a very

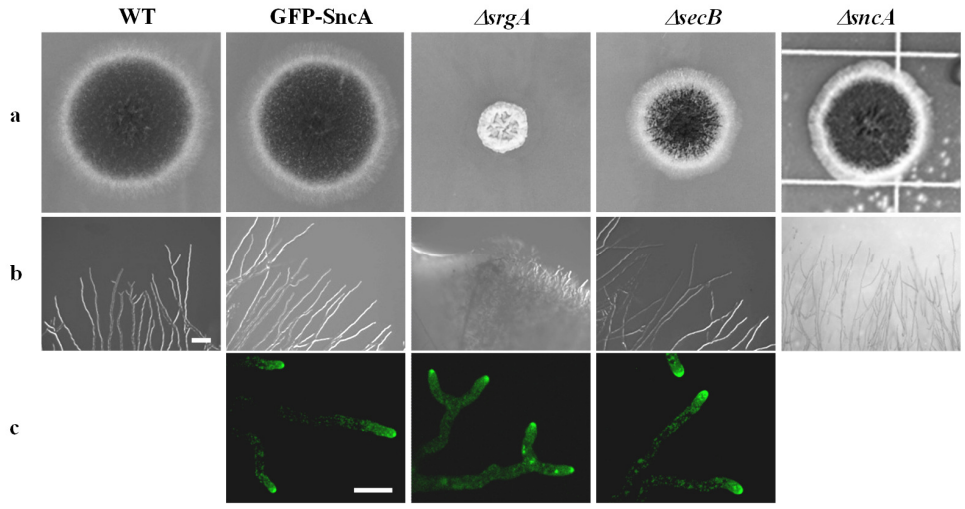

Fig. 3. Phenotypic analysis of *srgA secB* and *sncA* gene deletion mutants. a) Colony morphology of wild-type, GFP-SncA, *ΔsrgA*, *ΔsecB* and *ΔsncA* mutants after 3 days at 30°C on CM agar plates, b) hyphal morphology of the colony edge on CM agar plates (100x magnification, Bar, 100 µm) and c) localization of GFP-SncA in hyphal tip cells on MM agar plates. Bar, 10 µm.

compact colony after prolonged incubations at 30°C on minimal medium or on minimal medium supplemented with 1.2 M sorbitol. The primary transformants of the *ΔsecC* in the GFP-SncA background were not able to form colonies on minimal medium and the supplementation with sorbitol was required to obtain *ΔsecC* colonies. Although the replacement of SncA with GFP-SncA did not cause any growth-related phenotype (see above), the combination with the *secC* deletion was synthetic lethal, indicating that the function of SncA might partially be disturbed when fused to GFP. Interestingly, growth and germination of the *ΔsecC* mutant was improved by lowering the temperature to 22ºC (Fig. 4) but still partially unable to maintain polar growth as indicated by the presence of abnormally swollen hyphal tip cells. In agreement, the localization of GFP-SncA was highly affected in the *ΔsecC* mutant. Large fluorescent spots were present not only apically but also subapically, indicating that SecC is important for correct GFP-SncA localization at the hyphal apex. However, since GPF-SncA fluorescence was still preferentially localized at swollen hyphal tips, polarity was not completely lost in the *ΔsecC* mutant (Fig. 4b). The growth defect of the *ΔsecC* mutant was partially remediated by supplementing the growth medium with the osmotic stabilizer sorbitol, which was paralleled by partial repolarisation of GFP-SncA signals at hyphal tips (data not shown). The partial loss of polarization of GFP-SncA in the *secC* null mutant, indicates the importance of SecC for the maintenance of the polarity axis in growing *A. niger* hyphae (Fig. 4b).

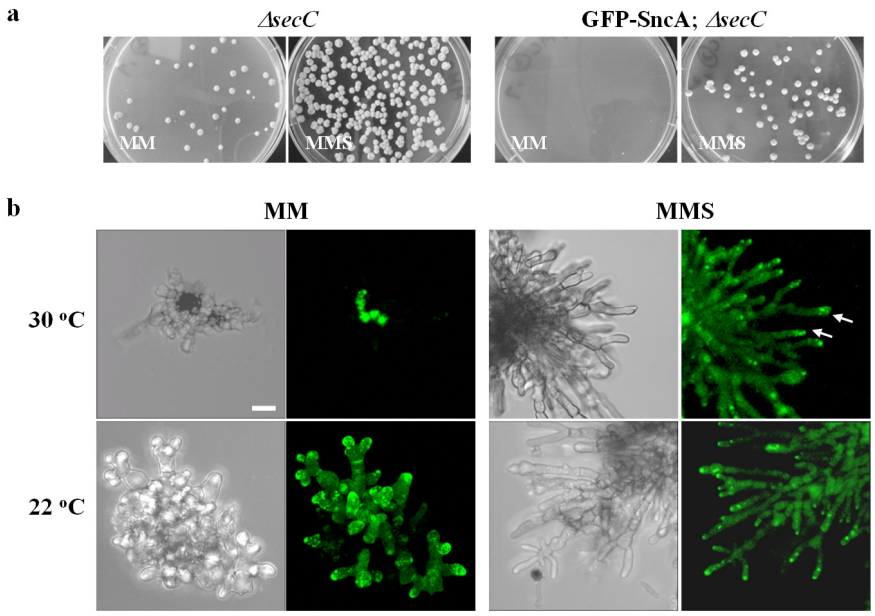

Fig. 4. Phenotypic analysis of *secC* gene deletion mutant. a) Deletions of *secC* in the wild-type and GFP-SncA background show severe growth defect that could be partially rescued by supplementation with 1.2 M Sorbitol (MMS). Deletion of *secC* in GFP-SncA shows a stronger growth defect than in a wild-type background. b) Microscopic picture of colony morphology and localization of GFP-SncA; *Δ*secC grown on MMS after 4 days at 30°C and 22°C. Arrows indicate GFP-SncA signals at the sub-apical region of the cell. Bar, 20 µm.

Point mutations in conserved residues of *A. niger ssoA* do not lead to ts-phenotype as in *S. cerevisae*

Conditional mutants are powerful tools to study gene functions (Li et al. 2011). To obtain a conditional mutant that accumulates secretory vesicles under restrictive temperature, the essential *ssoA* gene was chosen, which encodes the putative target-SNARE (t-SNARE) for fusion of Golgi-derived vesicles to the plasma membrane. In *S. cerevisiae,* temperature sensitive (ts) alleles of *sso1* or *sso2* have been described to result in conditional secretion mutants (Jantti et al., 2002). The protein amino acid sequence alignment of SsoA showed that this t-SNARE is highly conserved from budding yeast to mammals (Fig. 5). A site-directed mutagenesis approach was used to create *A. niger* strains that harbor point mutations in the *ssoA* gene causing a ts-phenotype in *S. cerevisiae*. The arginine to lysine mutation in *sso1* (R196K) or *sso2* (R200K) gives rise to a ts-phenotype (Jantti et al. 2002). As shown in Fig. 5, the arginine residue located at position 212 in the SsoA protein of *A. niger* is conserved from yeast to mammals. In addition, we also applied an algorithm to

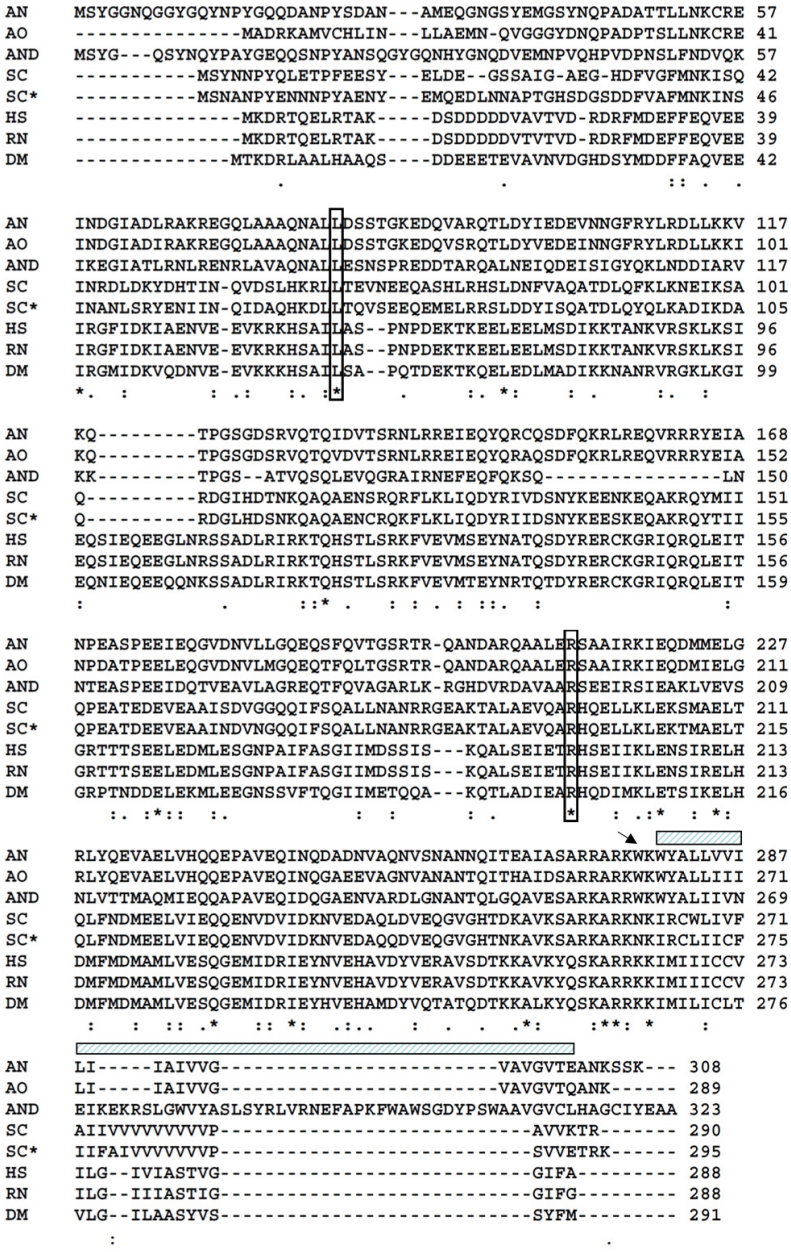

Fig. 5. Protein alignment of eukaryotic SsoA homologs. The amino acids which were chosen for site-directed mutagenesis are indicated by a box. The transmembrane domain is indicated by the hatched box above the sequence. The tryptophan at 278 aa was replaced to a stop codon to remove the transmembrane domain of SsoA; AN (*A. niger*, An12g01190), AO (*A. oryzae*, Q2TX29), AND (*A. nidulans*, Q5B7R4), SC (*S. cerevisiae*, Sso1p, P32867), SC* (*S. cerevisiae*, Sso2p, P39926), HS (*Homo sapiens*, Q16623), RN (*Rattus norvegicus*, P32851) and DM (*Drosophila melanogaster*, Q24547).

predict ts mutants based solely on the amino acid sequence (Varadarajan et al. 1996). The program identified a conserved leucine residue at position 81 as a preferred candidate. For both residues (L81 and R212), a conserved and a non-conserved mutation were created. The replacement cassettes consisting of four mutant alleles, L81F, L81G, R212K or R212P and one wild-type allele as a control were constructed and targeted to the *ssoA* locus to replace the resident *ssoA* gene as shown in Fig. 6a. Each of the mutants was verified by Southern blot analysis and the respective *ssoA* allele was re-sequenced from genomic DNAs of the transformants to verify correct replacement of the native *ssoA* gene with its point-mutated alleles (data not shown).

The leucine mutants, L81F and L81G, did not show any obvious phenotype at both 30°C or 37°C (Fig. 6b). Growth of the arginine mutant R212K was unaffected at 30°C, but slightly reduced at 37°C, whereby GFP-SncA fluorescence was still present apically (Fig. 6b). Replacing the arginine at codon 212 with a proline was lethal at both 30°C and 37°C (Fig. 6b), however, the strain survived when cultivated at lower temperature such as 22°C and 25°C (Fig. 6c). The growth defect of R212P mutant was partially complemented by supplementing the medium with sorbitol. Many large and round spots of GFP-SncA signals were observed inside swollen hyphae indicating an accumulation of secretory vesicles in the R212P mutant strain at 30°C (Fig. 6c). By supplementing sorbitol as well as by lowering the temperature, the R212P mutant was able to grow much better, but not as well as the wild-type strain. To examine whether one could use the R212P mutant to accumulate secretory vesicles, the *ssoA* R212P mutant was pre-grown at 22°C to allow the formation of young germlings and then shifted to 30°C for 6 hours. The temperature shift resulted in a variety of pleiotropic phenotypes such as accumulation of vesicles, increased septation, branching and formation of empty cell compartments (Fig. 6d). So although this approach resulted in temperature sensitive mutants, the phenotype of the mutants, either too mild (R212K) or to severe (R212P), did not allow their use to study the secretory pathway in more detail.

Controlled overexpression of SsoA lacking its transmembrane domain does not result in a conditional secretion mutant

Next, another approach to create a conditional mutant was tried by using the Tet-On system which we recently established for *A. niger* (Meyer et al., 2011). The SsoA protein contains a N-terminal syntaxin domain and C-terminal transmembrane domain (TM, 280 - 302 aa, Fig. 5). We aimed to establish a conditional SsoA mutant strain in which overexpression of a truncated SsoA version lacking the TM domain (*ssoA*ΔTM) disturbs fusion of secretory

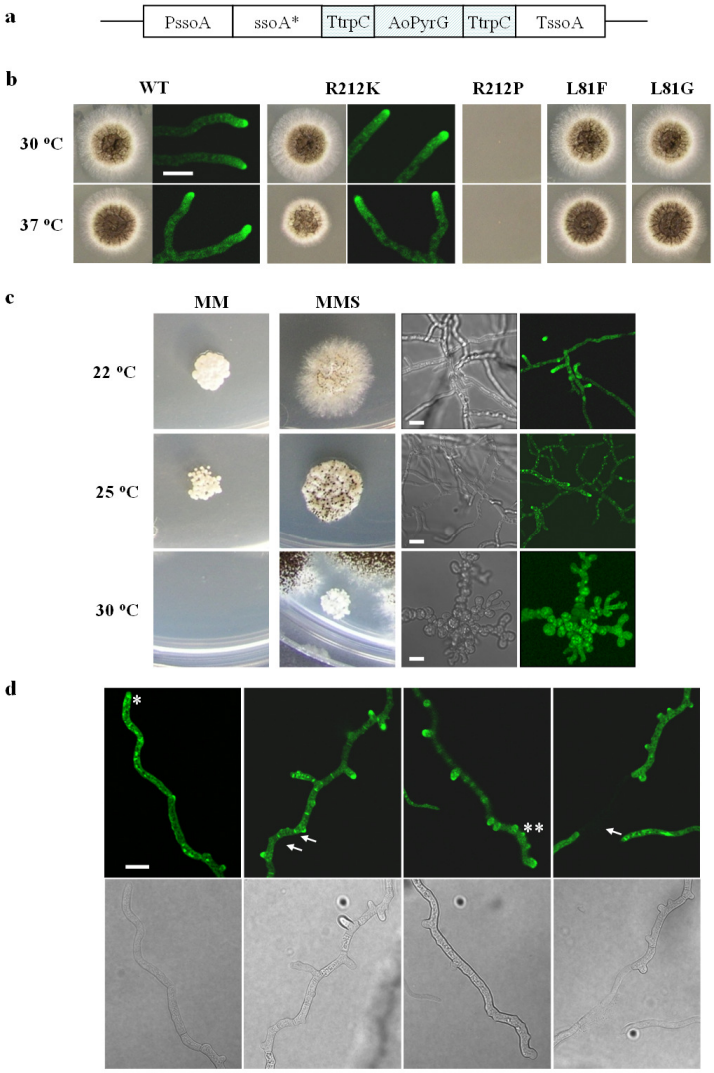

Fig. 6. Colony morphology and GFP-SncA localization in t-SNARE (*ssoA*) mutants.
a) Schematic representation of the approach to replace the wild-type t-SNARE protein (SsoA) with mutants forms of SsoA (labeled ssoA*). b) 400 spores were point inoculated on CM agar plates and incubated for 3 days at 30°C and 37°C. The R212K mutation in the *ssoA* gene leads to reduced growth at 37°C, but no apparent mislocalization of GFP-SncA; R212P mutation is lethal, whereas the L81F and L81G mutations have no apparent phenotype. Bar, 10 µm. c) Detailed growth analysis of the R121P mutant at various temperatures and in the presence of osmotic stabilizers (MMS). Low temperatures and high osmolarity conditions improve growth. At 30°C, hyphal growth and polarity as well as polarized localization of GFP-SncA is lost. Bar, 20 µm. d) Hyphal morphology and GFP-SncA localization of the R121P mutant after shifting from 22°C to 30°C for 6 hours. The temperature shift results in a variety of phenotypes as depicted; accumulated vesicles (*), increased septation (double arrows), increased branching (**), lysis and formation of empty compartments (a single arrow). Bar, 20 µm.

vesicles with the plasma membrane and thereby provoking accumulation of secretory vesicles. First, we confirmed that expression of *ssoA* under control of the P*tet* promoter (Ptet-ssoA-*pyrG**) did not affect growth and that P*tet* controlled expression of *ssoA* rescues the *ΔssoA* strain in a dose-dependent manner (Fig. 7 line a-c and see below). The importance of the TM domain for function of SsoA was verified by deleting the *ssoA* gene in a transformant that contained a Ptet-ssoAΔTM construct at the *pyrG* locus. In this strain, the TM of endogenous *ssoA* gene was removed by replacing it with a *ssoA* gene copy that contained an early stop codon at position 278 of the SsoA protein (Fig. 5). The inability to purify viable transformants in the absence or presence of DOX showed that the TM domain is indeed essential for the function of SsoA (Fig. 7 line e and data not shown). After transformation of the Ptet-ssoAΔTM cassette to the GFP-SncA reporter strain and verification of the correct integration by Southern blot analysis, the growth of *A. niger* and localization of GFP-SncA were examined by the addition of varying amounts of DOX to the growth medium. Unfortunately, overexpression of *ssoA*ΔTM did not result in a conditional mutant by interfering with growth of *A. niger* (Fig. 7 line f).

Controlled down-regulation of SsoA results in a conditional secretion mutant

A transformant containing the *ssoA* gene under the control of P*tet* promoter present in a *ΔssoA* background strain (MK34.1, Fig. 7 line c) was also analyzed by fluorescence microscopy (Fig. 7 line d). In the absence of DOX, most spores were able to germinate but soon after they lysed, empty germ tubes lacking GFP-SncA signals became visible. At very low DOX concentrations (0.2 µg/ml), cells were able to sustain growth, however, hyphal growth speed was considerably reduced and localization of GFP-SncA was severely affected before cells eventually lysed, demonstrating the importance of the t-SNARE SsoA not only for polarized growth and vesicular transport but also for maintaining cell wall integrity of the hyphal tip. Hyphal growth and apical GFP-SncA localization were completely reconstituted and comparable to the wild-type when DOX concentrations of 1.6 µg/ml or higher were added to the growth medium (Fig. 7 line c and d).

To examine whether it was possible to induce accumulation of secretory vesicles by the removal of DOX, spores of strain MK34.1 were germinated in the presence of 2.5 µg/ml DOX for 10 hours before the medium was replaced with DOX-free medium. Three hours after the removal of DOX, some of the germlings showed accumulated GFP-SncA signals or/and swollen hyphal tips (40%, n=10), but also germlings without morphological aberrations were found (Fig. 8a and data not shown). Microscopic analysis after 10 h of growth in the absence of DOX showed a heterogenic mixture of cells. About 25% of young

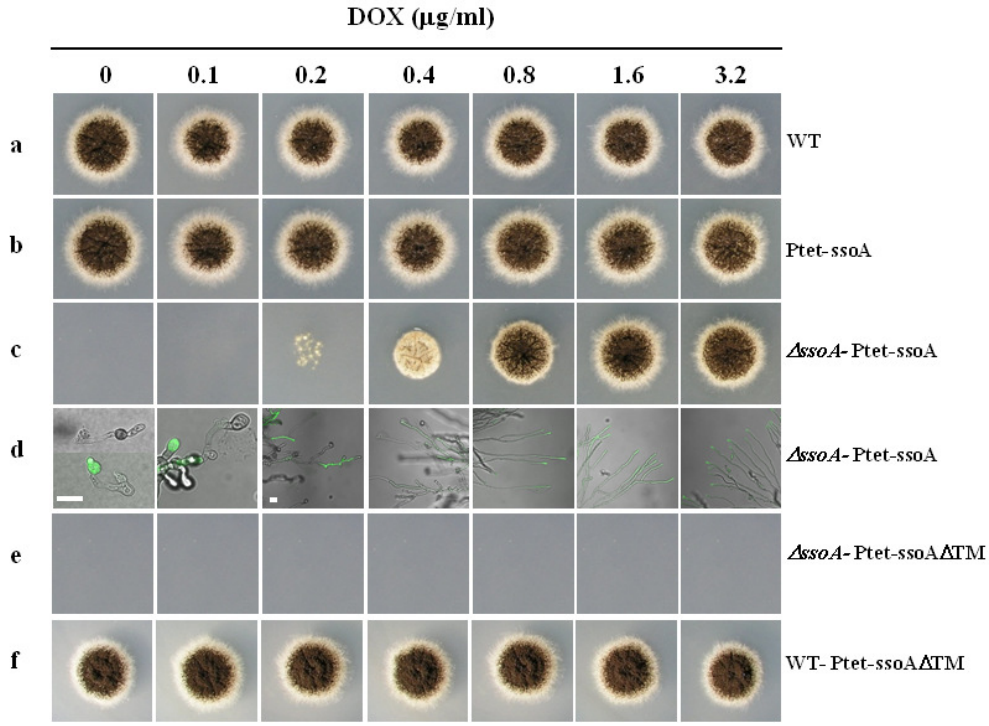

Fig. 7. Growth phenotype of strains overexpressing *ssoA* or *ssoAΔTM* in wild-type or *ΔssoA* background. a) Growth of the wild-type is not affected by the presence of DOX; b) overexpression of *ssoA* in wild-type background does not affect growth; c) the lethal phenotype of *ssoA* deletion can be rescued by controlled and tunable expression of *ssoA*; d) microscopic analysis of GFP-SncA localization in hyphae from the Δ*ssoA*-P*tet-ssoA* strain at various DOX concentrations; e) the transmembrane (TM) domain of SsoA is essential for growth; f) overexpression of the *ssoAΔTM* in wild-type strain does not interfere with growth; a-f) 10^3 spores were inoculated on MM supplemented with DOX concentrations indicated. Plates were cultivated for 3 days at 30°C. Bar, 20 µm.

germlings showed wild-type morphology, whereas the remaining germlings were characterized by swollen hyphal tips (Fig. 8b and data not shown). Apparently, residual intracellular amounts of SsoA were still present in some cells, which prevented a synchronous response of all germlings.

Discussion

In order to set the basis for systematic analysis of the protein secretion pathway in *A. niger*, we established a GFP-tagged vesicular SNARE reporter strain, GFP-SncA, to visualize the

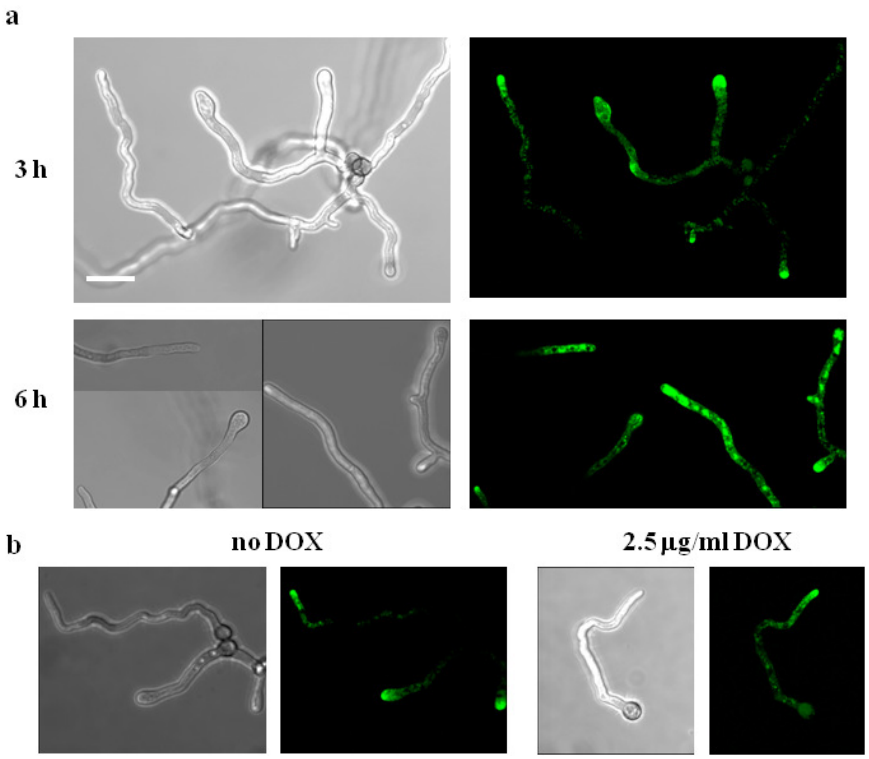

Fig. 8. Morphological phenotype and GFP-SncA localization resulting from controlled down-regulation of *ssoA*. a) Three and six hours after removal of DOX, accumulated GFP-SncA signals in swollen hyphal tips were observed, but also germlings without morphological aberrations were found. Spores were pre-grown on MM supplemented with 2.5 µg/ml of DOX at 30°C for 10 hours after which the medium was replaced with DOX free medium. b) Growth in the absence of DOX showed varying phenotypes after 10 hours of cultivation in DOX-free medium. Bar, 20 µm.

localization and dynamics of secretory vesicles. The localization of SncA homologs was reported in yeast as well as filamentous fungi including *S. cerevisiae*, *T. reesei*, *A. oryzae* and *A. nidulans* (Furuta et al. 2007; Hayakawa et al. 2011; Kuratsu et al. 2007; Taheri-Talesh et al. 2008; Valkonen et al. 2007). Similar to previous studies in filamentous fungi, GFP-SncA is present in intracellular structures representing secretory vesicles and/or endocytic vesicles. High levels of GFP-SncA are also present in the Spitzenkörper of *A. niger* and a tip gradient GFP-SncA localization was observed (Fig. 1). Occasionally, GFP-SncA signals were also observed at septa (data not shown), indicating the involvement of SncA in both hyphal tip secretion as well as septum-directed secretion that has recently been reported for SncA in *A. oryzae* (Hayakawa et al. 2011). In filamentous fungi, it is believed that the long distance transport of secretory vesicles from the sub-apical part to the

apex of hyphal tips takes place along microtubules (MT) powered by kinesin motor proteins. Afterwards, secretory vesicles are transferred either directly to the vesicle supplying center or to actin cables by myosin motor proteins and eventually fuse with the plasma membrane via SNARE complexes to release their cargos into the environment (Saloheimo and Pakula 2012; Steinberg 2007; Taheri-Talesh et al. 2008; Taheri-Talesh et al. 2012). The results shown in Fig. 2 support the importance of both the actin and the tubulin cytoskeletal elements for polarized transport and accumulation of secretory vesicles at hyphal tips of *A. niger*.

The GFP-SncA reporter strain was used to study the function of seven *A. niger* genes, whose orthologs are involved in vesicle transport in *S. cerevisiae*. Unlike *S. cerevisiae,* in which most of the selected candidate genes are essential for growth, *secB (SEC2)* and *sncA (SNC1)* genes are dispensable for *A. niger*, indicating molecular differences in the organization of secretion processes between yeast and filamentous fungi. Genetic redundancy in *A. niger* might explain this discrepancy, and further analysis of potential candidate genes exerting overlapping functions will require follow-up studies (see below).

It has previously been demonstrated that the *A. niger* SncA protein mediates the fusion of vesicles to the plasma membrane (Sagt et al. 2009). By fusing SncA to the peroxisome membrane using a peroxisomal anchor protein, peroxisomes were targeted to the plasma membrane where they fused with it, resulting in the secretion of peroxisomal cargoes (Sagt et al. 2009). Despite such an important cellular function, deletion of *sncA* had surprisingly only a very small effect on the growth of *A. niger*. A possible explanation for this might be the presence of alternative v-SNAREs, which are functionally redundant with SncA. However, all genome annotations of several filamentous fungi such as *T. reesei*, *Neurospora crassa*, *A. oryzae*, *A. nidulans* and *A. fumigatus* indicate that these fungi contain only a single copy of *sncA* in their genome (Gupta and Brent Heath 2002; Kienle et al. 2009; Kuratsu et al. 2007; Valkonen et al. 2007) as also reported for *A. niger* (Pel et al, 2007). Still, we considered the possibility of a redundant v-SNARE in the *A. niger* genome and searched the genome database for a SncA homolog (Table S2). A potential v-SNARE encoding gene with the highest level of similarity to SncA, and which contains a C-terminal synaptobrevin domain like SncA, is An08g07470 (47% identity, 66% similarity). An08g07470 contains a N-terminal longin domain (Wen et al. 2006) which is not present in SncA, thus making An08g07470 a larger protein (269 amino acids) than SncA (135 aa). Compared to *S. cerevisiae*, An08g07470 is most similar to the vacuolar v-SNARE component Nyv1p which is involved in homotypic vacuolar docking and fusion (Nichols et al. 1997). In *A. oryzae*, this v-SNARE is interestingly also localized to the plasma membrane, although to a lesser extent than the Snc1p homolog (Kuratsu et al. 2007). Hence, An08g07470 of *A. niger* could encode a functionally redundant protein for SncA.

The *S. cerevisiae* Sec2p protein is the guanine exchange factor (GEF) for the secretion related Rab GTPase Sec4p and its function is well characterized (Itzen et al. 2007; Walch-Solimena et al. 1997). GEFs stimulate the exchange of GDP for GTP, thereby activating its corresponding GTPase. Based on the results obtained in *S. cerevisiae*, deletion of the Sec2p ortholog in *A. niger* (SecB) was expected to result in a similar phenotype as the Sec4p ortholog (SrgA). However, deletion of *secB* in *A. niger* resulted in an almost wild-type phenotype in terms of fast hyphal growth and hyphal morphology, which is different from the Δ*srgA* phenotype (Fig. 3). The situation in *A. niger* is already different from that in *S. cerevisiae* as in *A. niger* the Sec4p ortholog itself is not essential for growth as is Sec4p in *S. cerevisiae*. Unlike other protein families, GEFs for different Rab GTPases do not share much sequence identity, making it difficult to predict protein function from sequence data (Segev 2001b). GEFs are considered to be GTPase specific, however, there are examples showing that one GEF complex acts on two GTPases; e.g. the TRAPP complex acts as a GEF for both Ypt1p and Ypt31/32p in *S. cerevisiae* (Jones et al. 2000). We thus assume that the genome of *A. niger* might encode alternative Rab GEF(s) which could activate SrgA in the absence of SecB. A possible candidate protein is An15g06770 which contains a GDP/GTP exchange factor Sec2p domain (pfam06428).

Using the essential *A. niger ssoA* gene, orthologous to the *S. cerevisiae* plasma membrane t-SNAREs Sso1/2p, three approaches were followed to create a conditional vesicles transport mutant of *A. niger*. First, we tried to establish a temperature sensitive mutant based on introducing ts-alleles of Sso1/2p in the *A. niger* SsoA ortholog. Despite the high sequence similarity and conservation of amino acid residues, introduction of the respective point mutations in SsoA did not result in a useful phenotype as it was either too mild or too severe (Fig. 6). The second approach focused on the establishment of a mutant which accumulates secretory vesicles via induced overexpression of a truncated SsoA version lacking the essential TM-domain (SsoAΔTM). Several studies on SNARE-mediated membrane fusion including Sso or Snc proteins demonstrated the importance of the TM domain for facilitating membrane fusion through the interaction of these TM domains (Fdez et al. 2010; Grote et al. 2000; Langosch et al. 2007; Lu et al. 2008). In agreement, this study provides evidence that the TM domain of the *A. niger* SsoA is also essential for its function (Fig. 7 line e). However, our data also clearly shows that a Tet-On based overexpression of SsoAΔTM, does not affect growth of *A. niger* (Fig. 7 line f). Note that it is unlikely that Tet-On mediated expression is insufficient to induce overexpression of the endogenous *ssoA* expression, as it was previously shown that the Tet-On system enables expression levels similar to *gpdA* expression (Meyer et al., 2011), which in fact would be 80-fold higher than *ssoA* expression (data not shown).

The third approach followed a strategy in which SsoA was down-regulated in a controlled manner using the Tet-On system. For this purpose, a strain was generated, which expressed *ssoA* from the P*tet* promoter in a Δ*ssoA* background strain. The resulting strain (MK34.1) is only viable in the presence of DOX but not in its absence. A wash-out experiment showed that pre-cultivation in medium containing 2.5 µg/ml DOX followed by a shift into DOX-free medium resulted in a conditional mutant phenotype characterized by the accumulation of secretory vesicles in the cytosol (Fig. 8a). However, we also observed that the accumulation of secretory vesicles was highly heterogeneous among germlings - some displayed the mutant phenotype and some localized the secretory vesicles still apically. This heterogeneous phenotype might possibly be explained by remnant intracellular concentrations of DOX and/or SsoA, e.g. due to low turnover rates. In both cases, slight amounts of functional SsoA might be still present in some cells thus sustaining normal growth.

Supportive for this explanation is the observation that the concentration of DOX that was used to make the spore plates had an effect on the germination characteristics. Spores that were taken from a plate that contained 100 µg/ml DOX formed normal germlings after transfer into medium lacking DOX. Apparently, a high concentration of DOX in the spore plates results in high *ssoA* mRNA and/or SsoA protein levels, thereby allowing germination without further induction of *ssoA*. Likewise, we noted that the concentration of DOX in the medium used for the pre-growth affected the outcome of the wash out experiment. A DOX concentration of 20 µg/ml during pre-growth (instead of the 2.5 µg/ml as shown in Fig. 8) and subsequent transfer into DOX-free medium increased the time to observe a morphological effect of SsoA depletion dramatically.

Taken together, we showed that controlled downregulation of SsoA via the Tet-On expression system can be used to create a conditional vesicular transport mutant of *A. niger*. However, this strain can display a heterogenous phenotype, which can partially be adjusted by controlling DOX concentrations. Such a conditional mutant will be an important tool for further work to unravel the mechanisms that enable *A. niger* to be an efficient protein secretor.

Methods

Strains, culture conditions and molecular techniques

The *Aspergillus niger* strains used in this study are listed in Table 2. Strains were grown on minimal medium (MM) containing 1% (w v^{-1}) glucose as carbon source (Bennett and

Lasure 1991). Complete medium (CM) consists of MM with the addition of 0.1% (w v^{-1}) casamino acids and 0.5% (w v^{-1}) yeast extract. When required, plates were supplemented with uridine (10 mM), hygromycin (100 μg ml^{-1}), doxycycline (Dox, 1-100 μg ml^{-1}) or sorbitol (1.2 M). Transformation of *A. niger* and fungal chromosomal DNA isolation was performed as described (Meyer et al. 2010b).

Construction of deletion cassettes, mutant alleles and expression cassettes

Standard PCR and cloning procedures were used for the generation of all constructs (Sambrook & Russell, 2001). All PCR amplified DNA sequences and cloned fragments were confirmed by DNA sequencing (Macrogene). All primers used in this study are listed in Table S1 in the supplemental material. Successful deletions or correct integration of GFP-constructs or mutant alleles were verified by Southern analysis.

The GFP-SncA construct was made using a combination of fusion PCR approaches combined with the MultiSite Gateway Three-Fragment Vector Construction Kit (Invitrogen) according to the manufacturer's instructions. Firstly, five individual DNA fragments were amplified by PCR using the primers listed in Table S1. These fragments include two *sncA* promoter regions (~950 bp and ~800 bp in length), the *sncA* ORF and terminator region of *sncA* (~1.2 kb), the *A. oryzae pyrG (AopyrG)* fragment (~1.8 kb) and the GFP fragment (~700 bp). The construct is schematically depicted in Fig. 1. The *AopyrG* marker is flanked by two identical promoter regions of *sncA* which allows efficient looping out of the *AopyrG* marker (Meyer et al. 2010b), for subsequent transformations using the *AopyrG* marker. The first promoter fragment was fused to the *AopyrG* fragment and the second promoter fragment was fused to GFP by a fusion PCR. The GFP-SncA final expression cassette was constructed using the three fragments, promoter-AopyrG, promoter-GFP and *sncA* ORF and terminator by the MultiSite Gateway Three-Fragment Vector Construction Kit (Invitrogen).

Constructs to delete the *secA*, *secB*, *secC*, *secH*, *ssoA* or *sncA* gene were made as follows: respective 5' flanking sequences (~700 bp) were obtained as *Kpn*I-*Xho*I fragments and 3' flanking sequences (~700 bp) were obtained as *Hind*III-*Not*I fragments by PCR using genomic DNA from strain N402 as a template. The respective 5' region *Kpn*I-*Xho*I fragments, 3' *Hind*III-*Not*I fragments and a 1.7 kb *Hind*III-*Xho*I fragment from pAO4-13 (de Ruiter-Jacobs et al. 1989) containing *AopyrG* gene were cloned into the pBluscript-SK^+ backbone prepared by digestion with *Kpn*I and *Not*I. In the case of *sncA*, a 3.1 kb of the hygromycin resistance cassette isolated from pAN7-1 (Punt et al. 1987) was used to replace the *sncA* ORF. For *srgA* gene deletion cassette was kindly provided by Bernhard Seiboth, Vienna, Austria.

Table 2. Strains used in this work.

Strain	Relevant genotype	Source
N402	*cspA1* (derivative of ATCC9029)	(Bos et al. 1988)
AB4.1	*pyrG*$^{-}$	(van Hartingsveldt et al. 1987)
AO4.13	*pyrG*$^{+}$(derivative of AB4.1 containing *A. oryzae pyrG*)	(Kwon et al. 2011)
MA70.15	*ΔkusA pyrG*$^{-}$ (derivative of AB4.1)	(Meyer et al. 2007a)
MA169.4	*kusA::DR-amdS-DR pyrG*$^{-}$	(Carvalho et al. 2010)
MA234.1	*kusA::DR-amdS-DR pyrG*$^{+}$	This work
FG7	*ΔkusA pyrG*$^{+}$ *egfp::sncA* (derivative of MA70.15)	This work
MK27.1	*kusA::DR-amdS-DR pyrG*$^{+}$, *ΔsncA hyg*^{r} (derivative of MA234.1)	This work
MK12.1	*ΔkusA pyrG*$^{-}$ *egfp::sncA* (derivative of FG7)	This work
MA164.1	Heterokaryon *ΔkusA, secA/pyrG*$^{-}$, *ΔsecA/pyrG*$^{+}$	This work
MK19.1	*ΔkusA pyrG*$^{+}$ *ΔsecB egfp::sncA* (derivative of MK12.1)	This work
MA165.1	*ΔkusA pyrG*$^{+}$ *ΔsecB* (derivative of MA70.15)	This work
MK20.2	*ΔkusA pyrG*$^{+}$ *ΔsecC egfp::sncA* (derivative of MK12.1)	This work
MK16.2	*kusA::DR-amdS-DR pyrG*$^{+}$ *ΔsecC* (derivative of MA169.4)	This work
MK17.5	Heterokaryon *ΔkusA, secH/pyrG*$^{-}$, *ΔsecH/pyrG*$^{+}$	This work
MK18.A	*ΔkusA pyrG*$^{+}$ *ΔsrgA egfp::sncA* (derivative of MK12.1)	This work
MK15.A	*kusA::DR-amdS-DR pyrG*$^{+}$ *ΔsrgA* (derivative of MA169.4)	This work
MA168.5	Heterokaryon *ΔkusA, ssoA/pyrG*$^{-}$, *ΔssoA/pyrG*$^{+}$	This work
MK28.1	*ΔkusA pyrG*$^{+}$ *ssoA egfp::sncA* (derivative of MK12.1)	This work
MK29.3	*ΔkusA pyrG*$^{+}$ *ssoAL81F egfp::sncA* (derivative of MK12.1)	This work
MK30.1	*ΔkusA pyrG*$^{+}$ *ssoAL81G egfp::sncA* (derivative of MK12.1)	This work
MK31.2	*ΔkusA pyrG*$^{+}$ *ssoAR212K egfp::sncA* (derivative of MK12.1)	This work
MK32.2	*ΔkusA pyrG*$^{+}$ *ssoAR212P egfp::sncA* (derivative of MK12.1)	This work
MK22.3	*ΔkusA TetO7*::Pmin::*ssoA pyrG*$^{+}$ (derivative of MK12.1)	This work
MK24.20	*ΔkusA TetO7*::Pmin::*ssoAΔTM pyrG*$^{+}$ (derivative of MK12.1)	This work
MK34.1	*ΔkusA ΔssoA hyg*^{r} *TetO7*::Pmin::*ssoA pyrG*$^{+}$ (derivative of MK12.1)	This work
MK33.1	Heterokaryon *ΔkusA, ssoA/hyg*$^{r-}$, *ΔssoA/hyg*$^{r+}$ *TetO7*::Pmin:: *ssoAΔTM pyrG*$^{+}$ (derivative of MK12.1)	This work

The *PssoA::ssoA::TtrpC::AopyrG::TtrpC::TssoA* construct for generating mutant alleles of *ssoA* (encoding SsoA^{L81F}, SsoA^{L81G}, SsoA^{R212K} and SsoA^{R212P}) was made by PCR amplification and subsequent cloning of four fragments. The four fragments include the ~0.7 kb promoter of *ssoA* as a *Kpn*I-*Xho*I fragment, the ~1.2 kb ORF of *ssoA* as a *Xho*I-*Eco*RI fragment, a ~2.8 kb *Not*I-*Asc*I fragment containing *TtrpC-AopyrG-TtrpC* (TPT)

selection marker and a ~0.7 kb *Asc*I-*Not*I fragment containing the terminator region of *ssoA*. The *TtrpC* repeats allow efficient loop out of the *pyrG* marker to allow subsequent transformations with the *pyrG* marker. Firstly, the 0.7 kb *Kpn*I-*Xho*I promoter fragment was cloned into the pBluscript-SK$^+$ backbone prepared by digestion with *Kpn*I and *Xho*I resulting pMK1. Then the fragments *NotI-Asc*I TPT and the *Asc*I-*Not*I terminator of *ssoA* were cloned into *Not*I restricted pMK1 to give pMK2. Finally, the *Xho*I-*EcoR*I ORF of *ssoA* was cloned into *Xho*I-*EcoR*I restricted pMK2. Mutant alleles of *ssoA* (encoding SsoA^{L81F}, SsoA^{L81G}, SsoA^{R212K} and SsoA^{R212P}) were generated by PCR using primers carrying respective mutations and *Xho*I-*EcoR*I ends. The DNA fragments encoding the respective mutations were cloned into *Xho*I-*EcoR*I restricted pMK2.

For the construction of conditional *ssoA* overexpression using the Tet-on system, the *ssoA* ORF or *ssoA* ORF truncated transmembrane domain domain (*ssoAΔTM*) were cloned into pVG2.2 (Meyer et al. 2011a) and the resulting plasmid was transformed for targeted integration at the *pyrG* locus using the *pyrG** marker. After Southern analysis, strains were selected (MK22.3 and MK24.20) that contained with wild-type *ssoA* gene or *ssoAΔTM* gene at the *pyrG* locus under control of the tetracycline inducible promoter. These strains were also used to delete the *ssoA* gene. To do so, the *ssoA::pyrG* disruption cassette (see above) was altered by replacing *AopyrG* selection marker with the *Hind*III-*Xho*I hygromycin resistance cassette that was obtained from pAN7-1 (Punt et al. 1987).

Microscopy

Light microscopic pictures for the edge of the colony were captured using an Axioplan 2 (Zeiss) equipped with a DKC-5000 digital camera (Sony). For the light and fluorescence images for GFP-SncA, pictures were captured with 40x C-apochromatic objective on an inverted LSM5 microscope equipped with a laser scanning confocal system (Zeiss Observer). LSM5 was also equipped with an incubator to control the cultivation temperature. The observation conditions for the life-imaging of hyphae were the same as described previously (Kwon et al. 2011). For time-lapse microscopy, in total seven z stacks (0.8 μm interval) were taken in 60 s time intervals. The time-lapse movie showing 4 frames per second was assembled using ZEN2009 software (Zeiss).

For the DOX washout experiments, cells were grown and observed on chamber glass slides (Lab-Tek II Chamber #1.5 German Coverglass System) with 2.5 μg/ml of DOX for 10 h at 30^{o}C. Subsequently, the culture medium containing DOX was gently removed from the observation chamber with a transfer pipette and replaced with medium without DOX. This was repeated at least five times. For benomyl and latrunculin B treatments, cells were grown on MM agar plate for 2 days at 22°C to avoid sporulation. The mycelium was cut

with a scalpel, placed upside down on a cover glass bottom culture dish containing one drop of MM containing 5 µg/ml benomyl or 2 µg/ml latrunculin B respectively, and incubated at 22°C for an additional hour before microscopically examined.

Acknowledgements

We acknowledge Sjors Noteboom for his contributions to cloning experiments and Cees van den Hondel for helpful and stimulating discussions. This project was carried out within the research programme of the Kluyver Centre for Genomics of Industrial Fermentation which is part of the Netherlands Genomics Initiative / Netherlands Organization for Scientific Research.