

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

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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* orthologues of seven secretion-specific genes, known to function in key aspects of the protein secretion machinery in *Saccharomyces cerevisiae*, was analysed 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 orthologue 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 *S. cerevisiae* is lethal. Similarly, deletion of the *A. niger* orthologue 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* (encoding SecA, SecH and SsoA the *A. niger* orthologues of *S. cerevisiae* Sec1p, Sec8p and Sso1/2p, respectively) showed that these genes are essential for *A. niger*, similar 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*.

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INTRODUCTION

As a member of the black aspergilli, *Aspergillus niger* is an important industrial micro-organism. It is used for the production of various food ingredients, pharmaceuticals and industrial enzymes (Fleissner & Dersch, 2010; Meyer, 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* in order to obtain insights into the molecular basis of these special properties (Carvalho *et al.*, 2010; Fleissner & Dersch, 2010; Jacobs *et al.*, 2009; Meyer, 2008; Meyer *et al.*, 2007a, 2010, 2011b;

Pel *et al.*, 2007). Using these tools, more complex processes such as the protein secretion process can also now be systematically 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 *Aspergillus nidulans*) or *Saccharomyces 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, were not known. Growth and secretion are considered to be tightly connected processes. Experiments in *A. niger* in chemostat cultures grown at identical growth

Abbreviations: DOX, doxycycline; SNARE, soluble NSF attachment protein receptor; ts, temperature sensitive.

Two supplementary tables, two supplementary figures and a video are available with the online version of this paper.

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 than on xylose at identical growth rates (Jørgensen *et al.*, 2009). One possible mechanism to explain this uncoupling of growth and secretion in *A. niger* would 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 & 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 [soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein receptor] complexes in the fungal tip cell (Valkonen *et al.*, 2007).

The secretion process involves an ordered transport of proteins via various organelles that 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 orthologue of Sec4p, was described in the past as being involved in protein secretion but not essential for the viability of *A. niger* (Punt *et al.*, 2001). Another secretion-related GTPase, SrgC, an orthologue of Rab6/Ypt6, was recently described as being required for maintaining the integrity of Golgi equivalents in *A. niger* (Carvalho *et al.*, 2011).

Other important factors involved in the secretion pathway as mediators of vesicle docking and fusion with the membrane are SNAREs (Bonifacino & Glick, 2004; Chen & Scheller, 2001). Like the Ypt/Rab proteins, these proteins are highly conserved in eukaryotic cells and most SNAREs are C-terminally anchored transmembrane proteins present on vesicle (v-SNAREs) and target (t-SNAREs) membranes (Bonifacino & Glick, 2004; Chen & Scheller, 2001; Gupta & 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-SNAREs are further subclassified into Qa, Qb and 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 helix complex called the SNARE complex at each fusion site (Bonifacino & Glick, 2004). The localization of SNARE proteins has been systematically analysed in *Aspergillus 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 has 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. Vesicle fusion 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 with Rho-GTPases and phosphatidylinositol 4,5-bisphosphate (PIP2) (for a recent review see Heider & Munson, 2012).

The availability of temperature-sensitive (ts) secretion mutants in *S. cerevisiae* has formed a strong basis for the 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 vesicles 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 are not essential in *A. niger* but are essential in *S. cerevisiae* indicates differences in the molecular mechanisms underlying the protein secretion process. For the essential *ssmA* gene, several approaches were undertaken to create a conditional secretion mutant of *A. niger*. Whereas attempts to introduce conserved ts mutations of the *S. cerevisiae* Sso1/Sso2p in the *A. niger* SsoA orthologue failed, a conditional *ssmA* mutant was obtained by controlled expression of *ssmA* in a *ssmA* deletion strain. Such a strain will facilitate synthetic lethal screens and the identification of high-copy-number suppressors in future secretion-related studies.

METHODS

Strains, culture conditions and molecular techniques. The *A. niger* strains used in this study are listed in Table 1. Strains were grown on minimal medium (MM) containing 1% (w/v) glucose as the carbon source (Bennett & Lasure, 1991). Complete medium (CM) consists of MM with the addition of 0.1% (w/v) casamino acids and 0.5% (w/v) 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 were performed as described previously (Meyer *et al.*, 2010).

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

Table 1. Strains used in this work

Strain	Relevant genotype	Source
N402	<i>cspA1</i> (derivative of ATCC 9029)	Bos <i>et al.</i> (1988)
AB4.1	<i>pyrG</i> ⁻	Van Hartingsveldt <i>et al.</i> (1987)
AO4.13	<i>pyrG</i> ⁺ (derivative of AB4.1 containing <i>A. oryzae pyrG</i>)	Kwon <i>et al.</i> (2011)
MA70.15	$\Delta kusA pyrG$ ⁻ (derivative of AB4.1)	Meyer <i>et al.</i> (2007a)
MA169.4	<i>kusA::DR-amdS-DR pyrG</i> ⁻	Carvalho <i>et al.</i> (2010)
MA234.1	<i>kusA::DR-amdS-DR pyrG</i> ⁺	This work
FG7	$\Delta kusA pyrG$ ⁺ <i>egfp::sncA</i> (derivative of MA70.15)	This work
MK27.1	<i>kusA::DR-amdS-DR pyrG</i> ⁺ $\Delta sncA hyg$ ^r (derivative of MA234.1)	This work
MK12.1	$\Delta kusA pyrG$ ⁻ <i>egfp::sncA</i> (derivative of FG7)	This work
MA164.1	Heterokaryon $\Delta kusA secA/pyrG$ ⁻ $\Delta secA/pyrG$ ⁺	This work
MK19.1	$\Delta kusA pyrG$ ⁺ $\Delta secB egfp::sncA$ (derivative of MK12.1)	This work
MA165.1	$\Delta kusA pyrG$ ⁺ $\Delta secB$ (derivative of MA70.15)	This work
MK20.2	$\Delta kusA pyrG$ ⁺ $\Delta secC egfp::sncA$ (derivative of MK12.1)	This work
MK16.2	<i>kusA::DR-amdS-DR pyrG</i> ⁺ $\Delta secC$ (derivative of MA169.4)	This work
MK17.5	Heterokaryon $\Delta kusA secH/pyrG$ ⁻ $\Delta secH/pyrG$ ⁺	This work
MK18.A	$\Delta kusA pyrG$ ⁺ $\Delta srgA egfp::sncA$ (derivative of MK12.1)	This work
MK15.A	<i>kusA::DR-amdS-DR pyrG</i> ⁺ $\Delta srgA$ (derivative of MA169.4)	This work
MA168.5	Heterokaryon $\Delta kusA ssoA/pyrG$ ⁻ $\Delta ssoA/pyrG$ ⁺	This work
MK28.1	$\Delta kusA pyrG$ ⁺ <i>ssoA egfp::sncA</i> (derivative of MK12.1)	This work
MK29.3	$\Delta kusA pyrG$ ⁺ <i>ssoAL81F egfp::sncA</i> (derivative of MK12.1)	This work
MK30.1	$\Delta kusA pyrG$ ⁺ <i>ssoAL81G egfp::sncA</i> (derivative of MK12.1)	This work
MK31.2	$\Delta kusA pyrG$ ⁺ <i>ssoAR212K egfp::sncA</i> (derivative of MK12.1)	This work
MK32.2	$\Delta kusA pyrG$ ⁺ <i>ssoAR212P egfp::sncA</i> (derivative of MK12.1)	This work
MK22.3	$\Delta kusA tetO7::Pmin::ssoA pyrG$ ⁺ (derivative of MK12.1)	This work
MK24.20	$\Delta kusA tetO7::Pmin::ssoA\Delta TM pyrG$ ⁺ (derivative of MK12.1)	This work
MK34.1	$\Delta kusA \Delta ssoA hyg$ ^r <i>tetO7::Pmin::ssoA pyrG⁺ (derivative of MK12.1)</i>	This work
MK33.1	Heterokaryon $\Delta kusA ssoA/hyg$ ^r $\Delta ssoA/hyg$ ^r <i>tetO7::Pmin::ssoA\Delta TM pyrG⁺ (derivative of MK12.1)</i>	This work

PCR-amplified DNA sequences and cloned fragments were confirmed by DNA sequencing (Macrogen). All primers used in this study are listed in Table S1, available in the online Supplementary Material. Successful deletions or correct integration of GFP constructs or mutant alleles were verified by Southern blotting.

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. Five individual DNA fragments were amplified by PCR using the primers listed in Table S1. These fragments included two *sncA* promoter regions (~950 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.*, 2010), 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 with 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 *KpnI*-*XhoI* fragments and 3' flanking sequences (~700 bp) were obtained as *HindIII*-*NotI* fragments by PCR using genomic DNA

from strain N402 as a template. The respective 5' region *KpnI*-*XhoI* fragments, 3' *HindIII*-*NotI* fragments and a 1.7 kb *HindIII*-*XhoI* fragment from pAO4-13 (de Ruiter-Jacobs *et al.*, 1989) containing the *AopyrG* gene were cloned into the pBluscript-SK⁺ backbone prepared by digestion with *KpnI* and *NotI*. In the case of *sncA*, 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*, a gene deletion cassette was kindly provided by Bernhard Seiboth, Institute of Chemical Engineering, University of Technology of Vienna, Vienna, Austria.

The *PsoA::ssoA::TtrpC::AopyrG::TtrpC::TsoA* 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 included the ~0.7 kb promoter of *ssoA* as a *KpnI*-*XhoI* fragment, the ~1.2 kb ORF of *ssoA* as a *XhoI*-*EcoRI* fragment, a ~2.8 kb *NotI*-*AscI* fragment containing *TtrpC*-*AopyrG*-*TtrpC* (TPT) selection marker and an ~0.7 kb *AscI*-*NotI* 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 *KpnI*-*XhoI* promoter fragment was cloned into the pBluscript-SK⁺ backbone prepared by digestion with *KpnI* and *XhoI*, resulting in pMK1. Then the fragments *NotI*-*AscI* TPT and the *AscI*-*NotI* terminator of *ssoA* were cloned into *NotI* restricted pMK1 to give pMK2. Finally, the *XhoI*-*EcoRI* ORF of *ssoA* was cloned into *XhoI*-*EcoRI* restricted pMK2. Mutant alleles of *ssoA* (encoding SsoA^{L81F}, SsoA^{L81G}, SsoA^{R212K} and SsoA^{R212P}) were generated by PCR using primers carrying the respective mutations and *XhoI*-*EcoRI* ends. The

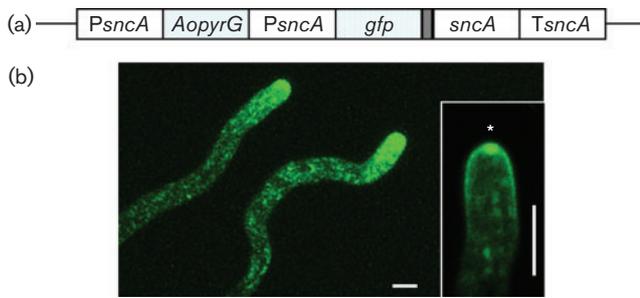


Fig. 1. Localization of v-SNARE protein SncA in living cells of *A. niger*. (a) Schematic representation of the approach used to label v-SNARE protein SncA with GFP. The GFP part was fused to the N-terminus of SncA and separated from SncA by a NPAFLYKVG-linker derived from Gateway cloning technology. The construct is designed for integration at the *sncA* locus using *A. oryzae pyrG* (*AopyrG*) as a selection marker. The promoter region in front of the DNA encoding the GFP-SncA fusion protein is about 800 bp to allow proper transcription and no interference of the *pyrG* gene. (b) CLSM 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 labelling of the plasma membrane. A clear gradient of GFP-SncA labelling towards the tip is visible. The Spitzenkörper is indicated with an asterisk. Bars, 5 μm .

DNA fragments encoding the respective mutations were cloned into *XhoI-EcoRI* restricted pMK2.

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

Microscopy. Light microscopy pictures of the edges of colonies 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 a $\times 40$ C-apochromatic objective on an inverted LSM5 microscope equipped with a laser scanning confocal system (Zeiss Observer). The LSM5 microscope was also equipped with an incubator to control the cultivation temperature. The observation conditions for the live-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 at 60 s time intervals. The time-lapse movie showing 4 frames s^{-1} was assembled using ZEN2009 software (Zeiss).

For the DOX wash-out experiments, cells were grown and observed on Chamber glass slides (Lab-Tek II Chamber no. 1.5 German coverglass system) with culture medium containing 2.5 μg DOX ml^{-1} for 10 h at 30 $^{\circ}\text{C}$. 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 plates for 2 days at 22 $^{\circ}\text{C}$ 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 benomyl ml^{-1} or 2 μg latrunculin B ml^{-1} , and incubated at 22 $^{\circ}\text{C}$ for an additional hour before being microscopically examined.

RESULTS

Localization of secretory vesicles in *A. niger*

The polarized delivery of secretory vesicles to the hyphal tips involves SNARE proteins as mediators of the vesicles docking and fusing with the plasma membrane. SNARE proteins are organelle specific, thereby ensuring the fusion of a vesicle to the correct target membrane (Chen & 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 homologue 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 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 favoured over C-terminal tagging, as the C-terminal transmembrane domain is required for proper localization and function of SNAREs (Taheri-Talesh *et al.*, 2008; Ungar & 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 analysed by Southern blot 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 analysed 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 centre (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, Supplementary video S1) as described

for *A. nidulans* and *A. oryzae* (Taheri-Talesh *et al.*, 2008, Kuratsu *et al.*, 2007). To examine the role of the tubulin and actin cytoskeleton in the localization of secretory vesicles, the GFP-SncA reporter strain was treated with benomyl and latrunculin B, respectively, known to disrupt the 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 (Fig. S2). 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 were selected (Table 2). These proteins were all selected based on high

amino acid sequence similarities with *S. cerevisiae* homologues (Pel *et al.*, 2007; Table S2). Expression analysis of the different genes confirmed that all chosen genes were actively expressed during germination and exponential growth (Table 2). 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 and in the GFP-SncA background. Primary transformants for *secA*, *secH* or *ssoA* survived only as heterokaryons containing transformed ($\Delta secA/pyrG^+$) 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 blot analysis (Fig. S1 and data not shown).

The growth phenotype and GFP-SncA localization of the viable deletion mutants were analysed 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 in the $\Delta srgA$ 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 the wild-type localization of secretory vesicles. SecB is the

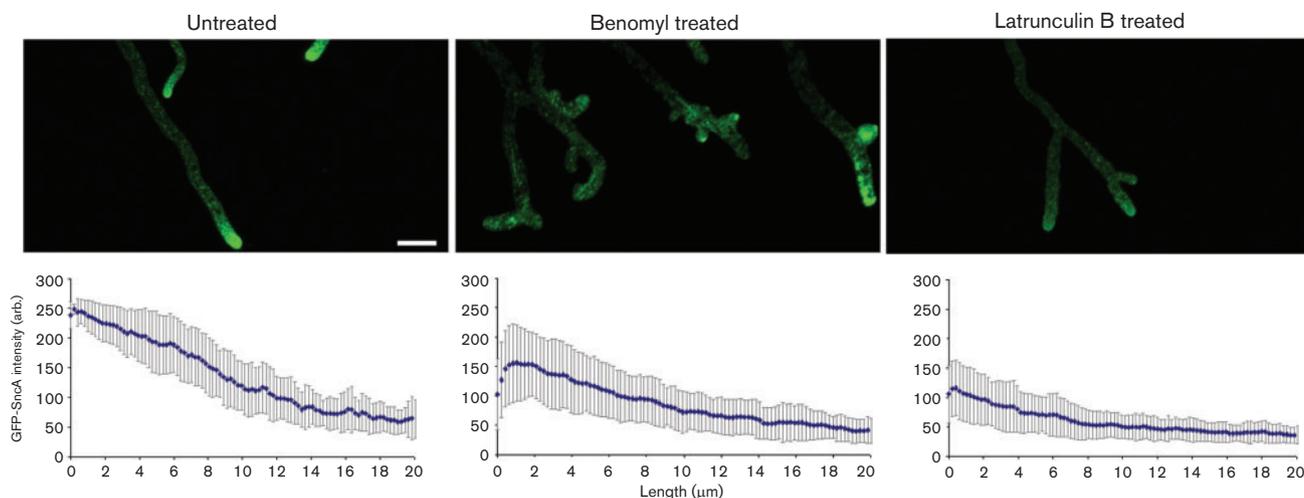


Fig. 2. Localization of v-SNARE protein SncA in living cells of *A. niger* after treatment with cytoskeleton-disturbing compounds. Top panels: CLSM images showing the localization of GFP-SncA in hyphal tips. Bars, 10 μm . Lower panels: fluorescence intensity distributions along hyphal tip compartments ($n > 20$) within a region of 20 μm from the tip (arb., arbitrary units). Conidia of the GFP-SncA strain were pre-grown on a MM agar plate for 2 days at 22 $^{\circ}\text{C}$ and placed on a cover glass bottom culture dish containing MM medium with 5 μg benomyl ml^{-1} or 2 μg latrunculin B ml^{-1} . After an additional hour of incubation, the cells were examined using an inverted confocal microscope.

Table 2. Expression and predicted function of selected secretion-related genes in *A. niger*

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

*Mean expression values are given as a percentage of the expression level of the actin-encoding gene *actA*. Data are taken from three independent cultivations. Exponential growth phase (Jørgensen et al., 2010); germination (Meyer et al., 2007b).

†*Sso1p* and *Sso2p*, as well as *Snc1p* and *Scn2p*, are paralogues and have a redundant function. Deletion of both genes is lethal in *S. cerevisiae* (Protopopov et al., 1993; Jännti et al., 2002).

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 $\Delta srgA$ phenotype. We thus examined whether another *sec2* homologue 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) that contains a GDP/GTP exchange factor Sec2p domain (pfam06428) (Table S2). It will be of interest to determine whether this hypothetical protein, which has orthologues in other filamentous fungi, has an overlapping role with SecB. In agreement with the mild phenotype of the $\Delta secB$ strain, the

localization of GFP-SncA in the $\Delta secB$ strain did not differ from the wild-type localization (Fig. 3).

The deletion of the v-SNARE encoding gene *sncA* resulted in a mild but significant phenotype with reduced radial growth (72 %) when compared with the wild-type strain (Fig. 3). Morphologically, the $\Delta sncA$ 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; Table S2).

In the case of $\Delta secC$, where *secC* is predicted to encode a subunit of the exocyst complex, the observed growth-defect

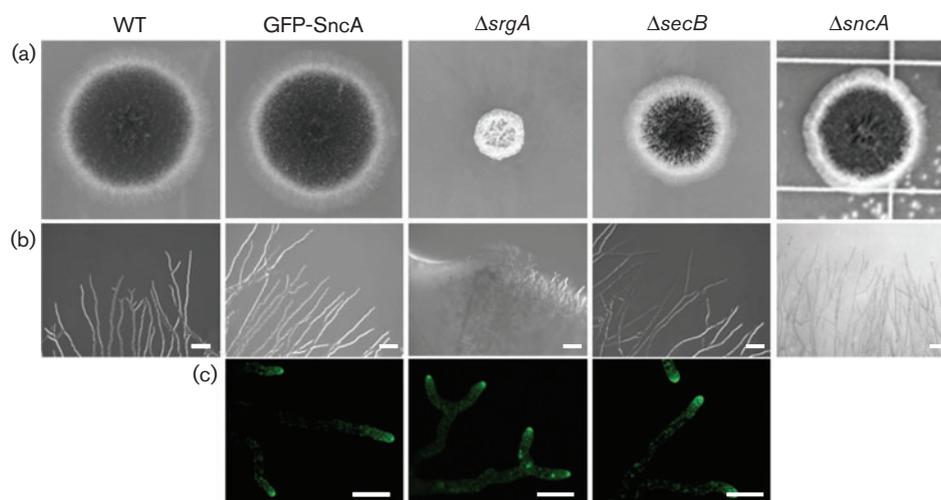


Fig. 3. Phenotypic analysis of *srgA*, *secB* and *sncA* gene deletion mutants. (a) Colony morphology of wild-type, GFP-SncA, $\Delta srgA$, $\Delta secB$ and $\Delta sncA$ mutants after 3 days at 30 °C on CM agar plates ($\times 0.5$ magnification). (b) Hyphal morphology of the colony edge on CM agar plates. Bars, 100 μm . (c) Localization of GFP-SncA in hyphal tip cells on MM agar plates. Bars, 10 μm .

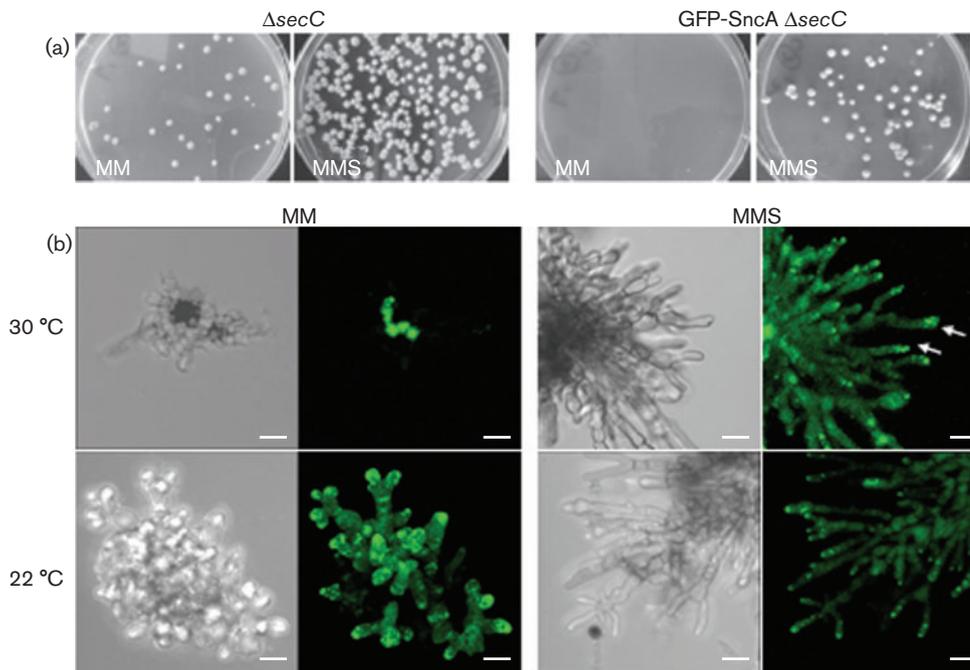


Fig. 4. Phenotypic analysis of the *secC* gene deletion mutant. (a) Deletions of *secC* in the wild-type and GFP-SncA background show severe growth defects that could be partially rescued by supplementation with 1.2 M sorbitol (MMS). Deletion of *secC* in GFP-SncA shows a stronger growth defect than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

phenotype was very severe and characterized by strongly reduced growth and aberrant morphologies of young germlings (Fig. 4a). The $\Delta secC$ strain in the wild-type background was able to grow on secondary selection plates only as a very compact colony after prolonged incubations at 30 °C on MM or on MM supplemented with 1.2 M sorbitol. The primary transformants of $\Delta secC$ in a GFP-SncA background were not able to form colonies on MM and the supplementation with sorbitol was required to obtain $\Delta secC$ 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 be partially disturbed when fused to GFP. Interestingly, growth and germination of the $\Delta secC$ mutant was improved by lowering the temperature to 22 °C (Fig. 4), but it was 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 $\Delta secC$ 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 GFP-SncA fluorescence was still preferentially localized at swollen hyphal tips, polarity was not completely lost in the $\Delta secC$ mutant (Fig. 4b). The growth defect of the $\Delta secC$ mutant was partially remediated by supplementing the

growth medium with the osmotic stabilizer sorbitol, which was paralleled by partial repolarization 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).

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

Conditional mutants are powerful tools for studying 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*, ts alleles of *sso1* or *sso2* have been described to result in conditional secretion mutants (Jäntti *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 harboured 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 (Jäntti *et al.*, 2002). As shown in Fig. 5, the arginine residue located at

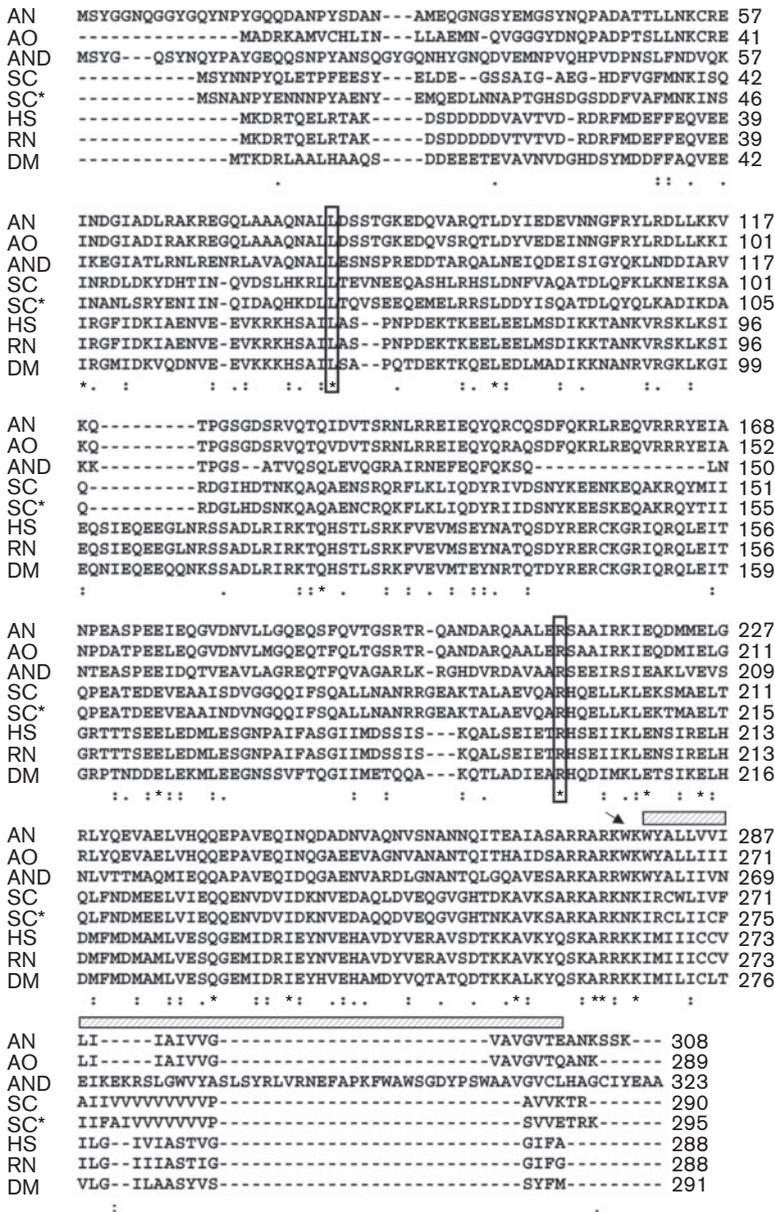


Fig. 5. Protein alignment of eukaryotic SsoA homologues. The amino acids that were chosen for site-directed mutagenesis are indicated by a box. The transmembrane domain is indicated by the hatched box above the sequence. The tryptophan at 278 aa was replaced with a stop codon to remove the transmembrane 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; DM, *Drosophila melanogaster* Q24547.

position 212 in the SsoA protein of *A. niger* is conserved from yeast to mammals. In addition, we also applied an algorithm to 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 resequenced 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 either 30 or 37 °C (Fig. 6b). Growth of the arginine mutant R212K was unaffected at 30 °C, but slightly reduced at 37 °C, when GFP-SncA fluorescence was still present apically (Fig. 6b). Replacing the arginine at codon 212 with a proline was lethal at both 30 and 37 °C (Fig. 6b); however, the strain survived when cultivated at lower temperatures such as 22 and 25 °C (Fig. 6c). The growth defect of the R212P mutant was partially complemented by supplementing the medium with sorbitol. Many large 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

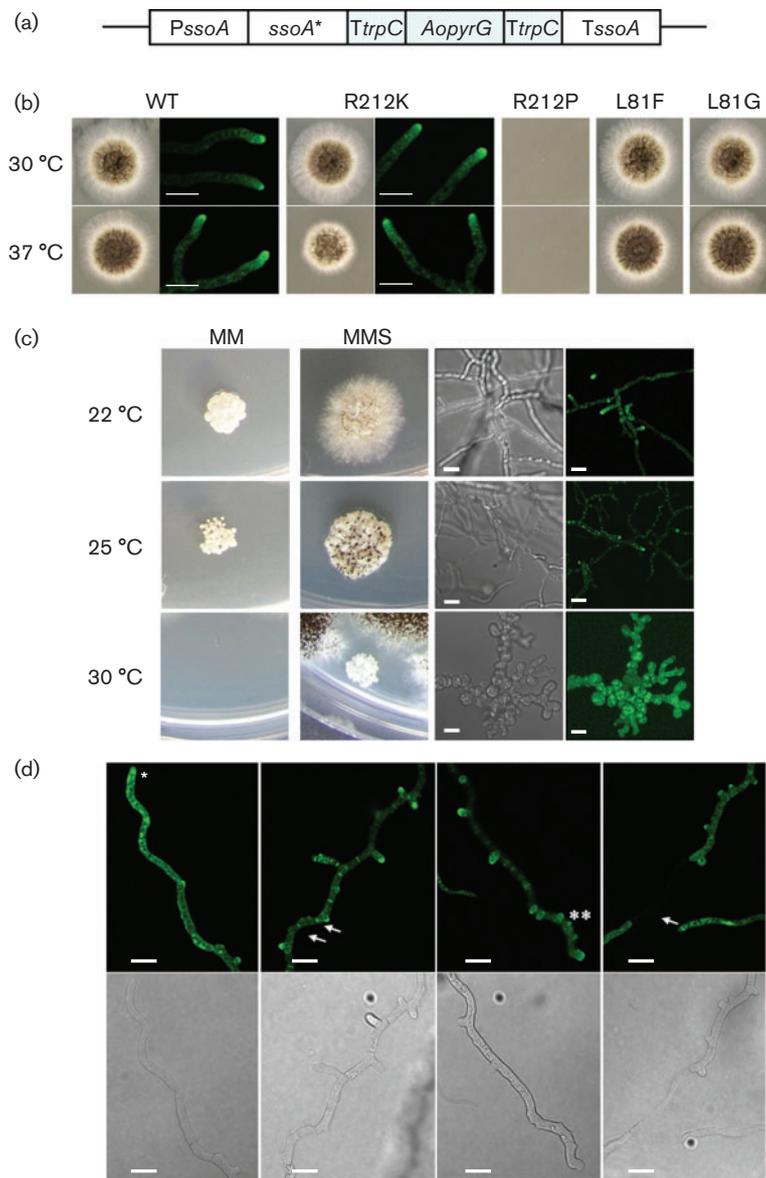


Fig. 6. Colony morphology and GFP-SncA localization in t-SNARE (*ssoA*) mutants. (a) Schematic representation of the approach used to replace the wild-type t-SNARE protein (SsoA) with mutant forms of SsoA (labelled *ssoA**). (b) Four hundred spores were point inoculated on CM agar plates and incubated for 3 days at 30 and 37 °C ($\times 0.25$ magnification). The R212K mutation in the *ssoA* gene leads to reduced growth at 37 °C, but no apparent mislocalization of GFP-SncA; the R212P mutation is lethal, whereas the L81F and L81G mutations have no apparent phenotype. Bars, 10 μm . (c) Detailed growth analysis of the R212P mutant at various temperatures and in the presence of the osmotic stabilizer sorbitol (MMS) ($\times 0.25$ magnification). 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. Bars, 20 μm . (d) Hyphal morphology and GFP-SncA localization of the R212P mutant after shifting from 22 to 30 °C for 6 h. 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 (single arrow). Bars, 20 μm .

whether the R212P mutant could 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 h. 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 ts mutants, the phenotype of the mutants, either too mild (R212K) or too 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 that we recently

established for *A. niger* (Meyer *et al.*, 2011a). The SsoA protein contains a N-terminal syntaxin domain and a C-terminal transmembrane domain (280–302 aa; Fig. 5). We aimed to establish a conditional SsoA mutant strain, in which overexpression of a truncated SsoA version lacking the transmembrane domain (SsoA ΔTM) disturbs fusion of secretory vesicles with the plasma membrane, thereby provoking accumulation of secretory vesicles. First, we confirmed that expression of *ssoA* under control of the *Ptet* promoter (*Ptet-ssoA-pyrG**) did not affect growth and that *Ptet*-controlled expression of *ssoA* rescues the ΔssoA strain in a dose-dependent manner (Fig. 7a–c, and see below). The importance of the transmembrane domain for function of SsoA was verified by deleting the *ssoA* gene in a transformant that contained a *Ptet-ssoA ΔTM* construct at the *pyrG* locus. In this strain, the transmembrane domain of the endogenous *ssoA* gene was removed and replaced with a *ssoA* gene

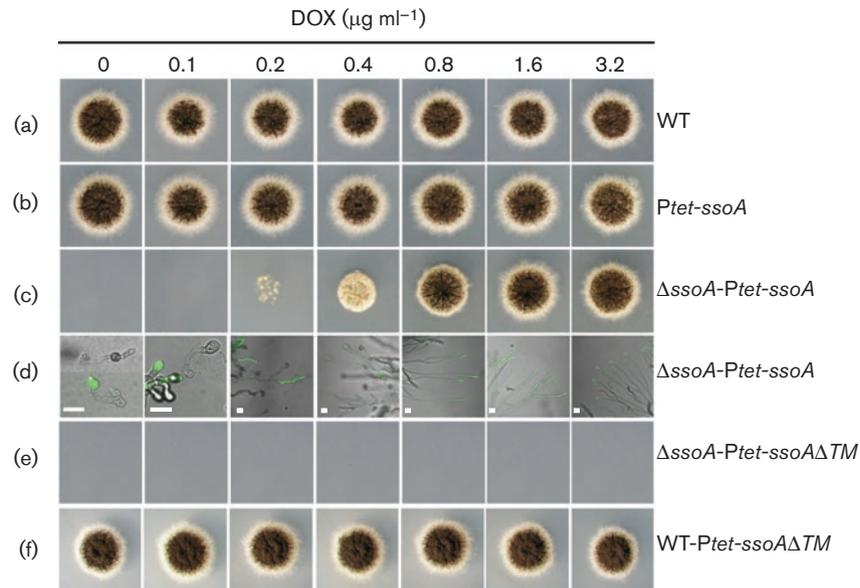


Fig. 7. Growth phenotype of strains overexpressing *ssoA* or *ssoA* Δ *TM* in the wild-type or Δ *ssoA* background. (a) Growth of the wild-type is not affected by the presence of DOX. (b) Overexpression of *ssoA* in the wild-type background does not affect growth. (c) The lethal phenotype of *ssoA* deletion can be rescued by controlled and tuneable expression of *ssoA*. (d) Microscopy analysis of GFP-SncA localization in hyphae from the Δ *ssoA*-*Ptet-ssoA* strain at various DOX concentrations. Bars, 20 μ m. (e) The transmembrane domain of SsoA is essential for growth. (f) Overexpression of the *ssoA* Δ *TM* in the wild-type strain does not interfere with growth. (a–f) Approximately 10^3 spores were inoculated on MM supplemented with the DOX concentrations indicated. Plates were cultivated for 3 days at 30 °C (magnification of colony pictures $\times 0.2$).

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 transmembrane domain is indeed essential for the function of SsoA (Fig. 7e; data not shown). After transformation of the GFP-SncA reporter strain with the *Ptet-ssoA* Δ *TM* cassette 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* Δ *TM* did not result in a conditional mutant by interfering with growth of *A. niger* (Fig. 7f).

Controlled downregulation of SsoA results in a conditional secretion mutant

A transformant containing the *ssoA* gene under the control of the *Ptet* promoter present in a Δ *ssoA* background strain (MK34.1; Fig. 7c) was also analysed by fluorescence microscopy (Fig. 7d). In the absence of DOX, most spores were able to germinate, but soon after they lysed, and empty germ tubes lacking GFP-SncA signals became visible. At very low DOX concentrations ($0.2 \mu\text{g ml}^{-1}$), 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 $1.6 \mu\text{g ml}^{-1}$ or higher were added to the growth medium (Fig. 7c, 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 $2.5 \mu\text{g DOX ml}^{-1}$ for 10 h 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; data not shown). Microscopic analysis after 10 h of growth in the absence of DOX showed a heterogeneous mixture of cells. About 25% of young germlings showed wild-type morphology, whereas the remaining germlings were characterized by swollen hyphal tips (Fig. 8b; 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

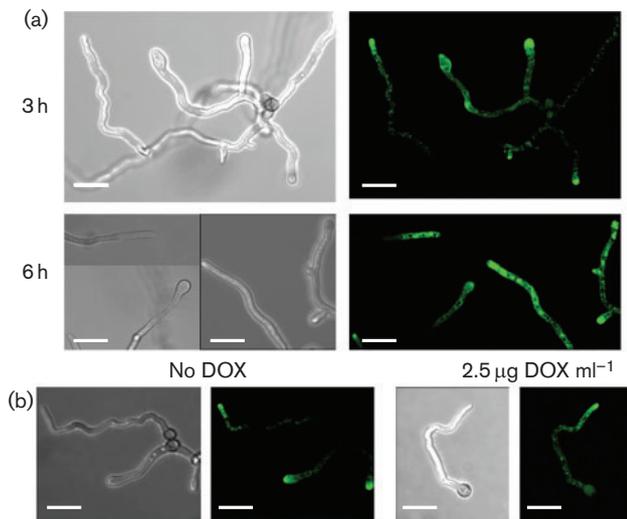


Fig. 8. Morphological phenotype and GFP-SncA localization resulting from controlled downregulation of *ssoA*. (a) Three and six hours after removal of DOX, accumulated GFP-SncA signals in swollen hyphal tips were observed, but germlings without morphological aberrations were also found. Spores were pre-grown on MM supplemented with 2.5 µg DOX ml⁻¹ at 30 °C for 10 h, after which the medium was replaced with DOX-free medium. (b) Growth in the absence of DOX showed varying phenotypes after 10 h of cultivation in DOX-free medium. Bars, 20 µm.

vesicular SNARE reporter strain, GFP-SncA, for visualizing the localization and dynamics of secretory vesicles. The localization of SncA homologues has been 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). Similarly 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 and septum-directed secretion, which 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 subapical part to the apex of hyphal tips takes place along microtubules powered by kinesin motor proteins. Afterwards, secretory vesicles are transferred either directly to the vesicle supplying centre or to actin cables by myosin motor proteins, and eventually fuse with the plasma membrane via SNARE complexes to release their cargoes into the environment (Saloheimo & Pakula, 2012; Steinberg, 2007; Taheri-Talesh *et al.*, 2008, 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 the hyphal tips of *A. niger*.

The GFP-SncA reporter strain was used to study the function of seven *A. niger* genes whose orthologues are involved in vesicle transport in *S. cerevisiae*. Unlike for *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 been demonstrated elsewhere 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 *Aspergillus fumigatus*, indicate that these fungi contain only a single copy of *sncA* in their genome (Gupta & 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 homologue (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 with *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 homologue (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 orthologue in *A. niger* (SecB) was expected to result in a similar phenotype to the Sec4p orthologue (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 orthologue 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 can act 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) that 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-encoding gene, orthologous to the *S. cerevisiae* plasma membrane t-SNARE Sso1/2p-encoding genes, three approaches were followed to create a conditional vesicle transport mutant of *A. niger*. First, we tried to establish a ts mutant based on introducing ts alleles of Sso1/2p in the *A. niger* SsoA orthologue. 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 that accumulates secretory vesicles via induced overexpression of a truncated SsoA version lacking the essential transmembrane domain (SsoA Δ TM). Several studies on SNARE-mediated membrane fusion including Sso or Snc proteins demonstrated the importance of the transmembrane domain for facilitating membrane fusion through the interaction of such 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 transmembrane domain of the *A. niger* SsoA is also essential for its function (Fig. 7e). However, our data also clearly show that Tet-On based overexpression of SsoA Δ TM does not affect growth of *A. niger* (Fig. 7f). Note that it is unlikely that Tet-On mediated expression is insufficient to induce overexpression of the endogenous *ssoA*, as it was previously shown that the Tet-On system enables expression levels similar to *gpdA* expression (Meyer *et al.*, 2011a), which in fact would be 80-fold higher than *ssoA* expression (data not shown).

The third approach followed a strategy in which SsoA was downregulated in a controlled manner using the Tet-On system. For this purpose, a strain was generated that expressed *ssoA* from the *Ptet* promoter in a Δ *ssoA* background strain. The resulting strain (MK34.1) was only viable in the presence of DOX, not in its absence. A wash-out experiment showed that pre-cultivation in medium containing 2.5 μ g DOX ml⁻¹ 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 still localized the secretory vesicles apically. This heterogeneous phenotype might possibly be explained by remnant intracellular concentrations of DOX and/or SsoA, for example, 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 μ g DOX ml⁻¹ 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 μ g ml⁻¹ during pre-growth (instead of 2.5 μ g ml⁻¹ as shown in Fig. 8) and subsequent transfer into DOX-free medium dramatically increased the time required to observe a morphological effect of SsoA depletion.

In summary, 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 heterogeneous phenotype, which can be partially 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.

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