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

Min Jin Kwon,1,2 Mark Arentshorst,1 Markus Fiedler,3 Florence L. M. de Groen,1 Peter J. Punt,1 Vera Meyer1,2,3 and Arthur F. J. Ram1,2

**Correspondence**
Arthur F. J. Ram
a.f.j.ram@biology.leidenuniv.nl

1Department Molecular Microbiology and Biotechnology, Institute of Biology Leiden, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands
2Kluvyer Centre for Genomics of Industrial Fermentation, P.O. Box 5057, 2600 GA Delft, The Netherlands
3Department Applied and Molecular Microbiology, Institute of Biotechnology, Berlin University of Technology, Gustav-Meyer-Allee 25, 13355 Berlin, Germany

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*.

**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...
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. glucanase) 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 & Bretschger, 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 exocyt is a conserved eukaryotic multimeric 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 ssoA 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 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.

**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
PCR-amplified DNA sequences and cloned fragments were confirmed by DNA sequencing (Macrogene). 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 fragment (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–Xhol 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–Xhol fragments, 3′ HindIII–NotI fragments and a 1.7 kb HindIII–Xhol fragment from pAO4-13 (de Ruiter-Jacobs et al., 1989) containing the AopyrG gene were cloned into the pBluescript-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 PssoA::ssoA::TrpC::AopyrG::TrpC::TssxA construct for generating mutant alleles of ssoA (encoding SsoAL81F, SsoAL81G, SsoAR212K and SsoAR212F) 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–Xhol fragment, the ~1.2 kb ORF of ssoA as a Xhol–EcoRI fragment, a ~2.8 kb NotI–AscI fragment containing TrpC–AopyrG–TrpC (TPT) selection marker and an ~0.6 kb AscI–NotI fragment containing the terminator region of ssoA. The TrpC repeats allow efficient loop out of the pyrG marker to allow subsequent transformations with the pyrG marker. Firstly, the 0.7 kb KpnI–Xhol promoter fragment was cloned into the pBluescript-SK+ backbone prepared by digestion with KpnI and Xhol, 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 Xhol–EcoRI ORF of ssoA was cloned into Xhol–EcoRI restricted pMK2. Mutant alleles of ssoA (encoding SsoAL81F, SsoAL81G, SsoAR212K and SsoAR212F) were generated by PCR using primers carrying the respective mutations and Xhol–EcoRI ends.

### Table 1. Strains used in this work

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<th>Strain</th>
<th>Relevant genotype</th>
<th>Source</th>
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<td>cspA1 (derivative of ATCC 9029)</td>
<td>Bos et al. (1988)</td>
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<td>AB4.1</td>
<td>pyrG−</td>
<td>Van Hartingsveldt et al. (1987)</td>
</tr>
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<td>A04.13</td>
<td>pyrG+ (derivative of AB4.1 containing A. oryzae pyrG)</td>
<td>Kwon et al. (2011)</td>
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<td>MA70.15</td>
<td>ΔkusA pyrG− (derivative of AB4.1)</td>
<td>Meyer et al. (2007a)</td>
</tr>
<tr>
<td>MA169.4</td>
<td>kusA::DR-amds-DR pyrG−</td>
<td>Carvalho et al. (2010)</td>
</tr>
<tr>
<td>MA234.1</td>
<td>kusA::DR-amds-DR pyrG−</td>
<td>This work</td>
</tr>
<tr>
<td>FG7</td>
<td>ΔkusA pyrG+ egfp::sncA (derivative of MA70.15)</td>
<td>This work</td>
</tr>
<tr>
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<td>This work</td>
</tr>
<tr>
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<td>MK34.1</td>
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</tr>
<tr>
<td>MK33.1</td>
<td>Heterokaryon ΔkusA ΔssoA/hyg′ ΔssoA/hyg′+ tetO7::Pmin::ssoAΔTM pyrG+ (derivative of MK12.1)</td>
<td>This work</td>
</tr>
</tbody>
</table>
DNA fragments encoding the respective mutations were cloned into XhoI–EcoRI restricted pMK2.

For the creation of a conditional ssoA overexpression construct using the Tet-On system, the ssoA ORF or ssoA ORF with a truncated transmembrane domain (ssoAAΔM) was cloned into pVG2.2 (Meyer et al., 2011a) and the resulting plasmid was transformed for targeted integration at the pyrG locus using the 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. After Southern blot analysis, strains were selected (MK22.3 and MK24.20) that contained the wild-type ssoA gene or ssoAAΔM gene at the pyrG locus under control of the tetracycline 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 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 ×40 C-achromatic 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 °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 °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 °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 SlA-B-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 (ΔsecA/ΔpyrG+) and untransformed nuclei (secA/ΔpyrG+) 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 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 the wild-type localization of secretory vesicles. SecB is the

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**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 °C and placed on a cover glass bottom culture dish containing MM medium with 5 μg benzoyl ml⁻¹ or 2 μg latrunculin B ml⁻¹. After an additional hour of incubation, the cells were examined using an inverted confocal microscope.
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 \(D_srgA\) 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 \(D_sncA\) 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

<table>
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<tr>
<th>(A.\ niger) gene</th>
<th>(S.\ cerevisiae) gene</th>
<th>(A.\ nidulans) gene</th>
<th>ORF code</th>
<th>Expression*</th>
<th>Predicted function</th>
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<td>(secA)</td>
<td>(SEC1)</td>
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<td>An14g03790</td>
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<td>(SEC2)</td>
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<td>(secC)</td>
<td>(SEC3)</td>
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<td>(SEC4)</td>
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<td>5.40 ± 0.23</td>
<td>1.97 ± 0.26</td>
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<td>–</td>
<td>An03g04210</td>
<td>0.69 ± 0.03</td>
<td>0.57 ± 0.02</td>
</tr>
<tr>
<td>(ssoA)</td>
<td>(SSO1/2)\†</td>
<td>(ssoA)</td>
<td>An12g01190</td>
<td>1.14 ± 0.14</td>
<td>1.71 ± 0.04</td>
</tr>
<tr>
<td>(sncA)</td>
<td>(SNC1/2)\†</td>
<td>(sncA)</td>
<td>An12g07570</td>
<td>5.18 ± 0.19</td>
<td>4.00 ± 0.03</td>
</tr>
</tbody>
</table>

*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).

\(\Delta Sso1p\) 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).

![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 (×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.](http://mic.sgmjournals.org)
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 compact colony after prolonged incubations at 30 °C on MM or on MM supplemented with 1.2 M sorbitol. The primary transformants of ΔsecC in a GFP-SncA background were not able to form colonies on MM 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 be partially disturbed when fused to GFP. Interestingly, growth and germination of the ΔsecC 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 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 GFP-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 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 (Jänntti *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 (Jänntti *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 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

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**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* P32861; DM, *Drosophila melanogaster* Q24547.
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.

Figure 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 (×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 R121P mutant at various temperatures and in the presence of the osmotic stabilizer sorbitol (MMS) (×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 R121P 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.

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.

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**Figure 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 (×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 R121P mutant at various temperatures and in the presence of the osmotic stabilizer sorbitol (MMS) (×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 R121P 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.
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-ssoAΔTM cassette and verification of the correct integration by Southern blot analysis, the growth of Aspergillus niger and localization of GFP-SncA were examined by the addition of varying amounts of DOX to the growth medium. Unfortunately, overexpression of ssoAΔTM did not result in a conditional mutant by interfering with growth (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 ΔssoA 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 μg 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 μg 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 μg 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
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 Spizetknker 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

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 germings 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. Bars, 20 μm. (b) Growth in the absence of DOX showed varying phenotypes after 10 h of cultivation in DOX-free medium. Bars, 20 μm.
from the \(\Delta\)srgA phenotype (Fig. 3). The situation in \textit{A. niger} is already different from that in \textit{S. cerevisiae} as in \textit{A. niger} the Sec4p orthologue itself is not essential for growth as is Sec4p in \textit{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 \textit{S. cerevisiae} (Jones et al., 2000). We thus assume that the genome of \textit{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 \textit{A. niger} SsoA-encoding gene, orthologous to the \textit{S. cerevisiae} plasma membrane t-SNARE Sso1/2p-encoding genes, three approaches were followed to create a conditional vesicle transport mutant of \textit{A. niger}. First, we tried to establish a ts mutant based on introducing ts alleles of Sso1/2p in the \textit{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\textsubscript{ATM}). 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 \textit{A. niger} SsoA is also essential for its function (Fig. 7e). However, our data also clearly show that Tet-On based overexpression of SsoA\textsubscript{ATM} does not affect growth of \textit{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 \textit{Pter} promoter in a \(\Delta\)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 \(\mu\)g DOX \textsubscript{ml\textsuperscript{-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 \(\mu\)g DOX \textsubscript{ml\textsuperscript{-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 \(\mu\)g ml\textsuperscript{-1} during pre-growth (instead of 2.5 \(\mu\)g ml\textsuperscript{-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 \textit{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 \textit{A. niger} to be an efficient protein secretor.

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Vesicle transport in Aspergillus niger


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