Role of the Zinc Finger Transcription Factor SltA in Morphogenesis and Sterigmatocystin Biosynthesis in the Fungus Aspergillus nidulans

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Abstract

Potassium, a widely accepted macronutrient, is vital for many physiological processes such as regulation of cell volume, maintenance of intracellular pH, synthesis of proteins and activation of enzymes in filamentous fungi. Another cation, calcium, plays an essential role in many signaling processes from lower to higher eukaryotes. Imbalance in the intracellular ionic levels of potassium or calcium causes adverse effects on cell growth, morphology and development, and eventually death. Previous studies on the adaptation of Aspergillus nidulans to salt and osmotic stress conditions have revealed the role of SltA, a C2H2 zinc finger transcription factor, in cation homeostasis. SltA is highly conserved in the Ascomycota phylum with no identifiable homolog in S. cerevisiae and other yeast-like fungi, and prevents toxicity by the cations Na⁺, K⁺, Li⁺, Cs⁺ and Mg²⁺, but not by Ca²⁺. However its role in morphology and biosynthesis of natural products such as mycotoxins remained unknown. This study shows the first characterization of the role of calcium and SltA fungal homologs in morphogenesis using the model system A. nidulans. Addition of potassium to sltA deletion mutants resulted in decreased levels of sterigmatocystin production. A similar phenotype was observed for both types of mutants in veA¹ and veA² genetic background. Expression of the sterigmatocystin genes aflR and stcU was strongly reduced in sltA deletion mutant when K⁺ was added. Additionally, increased concentrations of K⁺ drastically reduced sexual and asexual development, as well as radial growth in deletion sltA colonies. This reduction was accompanied by lower expression of the morphology related genes nsdD, steA and brlA. Interestingly, addition of calcium was able to stimulate asexual and sexual development and remediate the deletion sltA phenotype, including defects in morphology and toxin production.

Introduction

Filamentous fungi inhabit a wide range of both indoor and outdoor environments, which includes soil, plants, animals and human hosts. They are also capable of growing in extreme environmental niches such as salterns, hot springs, deserts, deep sea sediments, bird excreta, aquatic habitats, mine drainages and in the crevices of rocks. Every single environmental condition mentioned above presents various forms of stresses including osmotic stress, oxidative stress, nutrient deprivation, changes in pH and heat shock. Fungi have developed sophisticated mechanisms to alleviate the extracellular stress associated with these harsh conditions and thereby promote growth and survival.

Potassium is a very important nutrient required for many physiological processes such as regulation of cell volume, maintenance of intracellular pH, synthesis of proteins and activation of enzymes in several organisms such as plants, animals, bacteria and filamentous fungi. However, under hypertonic ambient conditions, high-salt concentrations cause cells to lose water when cations enter the cell. Na⁺ is highly toxic and is usually maintained in low levels, while K⁺ is accumulated at high concentrations in the cell. When the intracellular concentration of Na⁺ approaches that of K⁺, the cell growth is inhibited. Thus, in order to maintain cation homeostasis, specific transporters facilitate accumulation of K⁺ in the cytoplasm and extrusion of Na⁺ out of the cells. For more than five decades, yeast cells have been employed as model
systems to study the alkaline metal cation transport and homeostasis [1].

In *A. nidulans* the activity of a zinc-finger transcription factor, SltA, has been associated with tolerance to salt stress. SltA, also called StzA in some reports, was identified in early studies of salt tolerance in *A. nidulans* [2]. The *sltA* gene, located on chromosome VI in *A. nidulans* encodes a 698-amino acid-residue protein containing three classical Cys2His2 zinc fingers [3,4]. Phylogenetic distribution of this transcription factor is limited to the Pezyzomycotina phylum, subsequently with no identifiable homolog in *S. cerevisiae* [4,5]. O'Mahony et al., (2002) [6] showed that a *sltA1* mutant was defective in arginine activity, which also coincided with reduced salt tolerance in *A. nidulans*. Studies by O’Neil et al., 2002 [3] reported that besides exhibiting tolerance to salt stress, StzA also has an important role in DNA repair functions. The null allele of *sltA* causes sensitivity to elevated concentrations of monovalent cations such as sodium, lithium, rubidium and potassium, among others, and to divalent cations such as manganese and magnesium, but not to calcium [4].

Another important phenotype is the sensitivity of the null *sltA* strain to alkalinity, shared with null alleles of other general transcription factors such as *CrzA* [4], the regulator of calcium stress, and *PacC*, the well-known mediator of ambient pH regulation in *A. nidulans* [7,8]. ACE1 from *Trichoderma reesei*, a transcription factor with strong similarity to StzA that regulates expression of cellulase and xylanase promoters, binds to the consensus 5'-AGGCA-3' [9]. StzA also binds to this target, which has been found in genes under its regulation such as the vacuolar calcium exchanger,*vcxA* [4,10].

Despite this extensive work on the regulation of cation tolerance by StzA, nothing was known about its possible role in the regulation of fungal morphogenesis and secondary metabolism. *Aspergillus nidulans* efficiently disseminates by developing asexually formed air-borne conidia on specialized structures denominated conidiophores. Production of these structures is spatially and temporarily regulated [11,12]. Additionally, *A. nidulans* also develops fruiting bodies called cleistothecia where sexual spores, or ascospores, are formed [13]. Previous studies have revealed genetic regulatory links governing both morphogenesis and secondary metabolism in filamentous fungi [14]. For example, we have previously shown that the conserved global regulatory gene *veA*, initially known to control sexual and asexual development in *A. nidulans* [15], was also required for the synthesis of numerous secondary metabolites, including sterigmatocystin (ST) [16,17]. ST is a mycotoxin structurally similar to aflatoxins (AF), highly carcinogenic compounds produced by related *Aspergillus* species including *A. flavus*, *A. parasiticus*, and *A. nomiusi* [18–21]. Because both ST and AF are synthesized through a conserved biosynthetic pathway where ST is the penultimate precursor, and due to the fact that regulatory mechanisms controlling the production of both toxins tend to be conserved, *A. nidulans* has been used as an efficient model system in the study of AF/ST genetic regulation [22–26]. It is possible that elements involved in the regulation of cation homeostasis could also influence both morphogenesis and mycotoxin biosynthesis. In this study we showed that indeed *sltA*, in addition to its described role in cation stress tolerance, is also necessary for morphological development and sterigmatocystin production in *A. nidulans*.

**Materials and Methods**

**Strains and culture conditions**

The *Aspergillus nidulans* strains used in this study are listed in Table 1. Meiotic recombination was performed in order to obtain the null *sltA* mutant in a *veA* background from the original ∆*sltA* mutant carrying the *veA1* allele. The ∆*sltA*, *veA1* strain was initially obtained as previously described [4]. Essentially, the *sltA* coding region was precisely deleted by replacement with the *Aspergillus fumigatus* riboB gene. All strains generated by Spielvogel et al., 2008 [4] contained the *veA1* mutation. Genetic techniques were performed as previously described [27]. MAD1685 (*inoB2, ∆sltA::riboB, veA1*) was crossed with WIM126 (*pabaA1,yA2,veA*) and the resulting progenies were screened for *veA* genotype and confirmed by sequencing.

Strains were grown on solid glucose minimal medium, GMM [28], at 37°C unless otherwise indicated. Agar (10 g/liter) was added for solid medium. Strains were stored as glycerol stocks at -80°C.

**Fungal growth and developmental studies**

SltA deletion strains (with *veA1* or *veA*’ genetic background, HHF27B and RSS1.6P respectively) along with the corresponding prototrophic controls (HHF27A and FGSC4 respectively) were point inoculated on GMM, and GMM, supplemented with 200 mM or 400 mM KCl, unless otherwise indicated. Cultures were then incubated for 5 days at 37°C. Fungal growth was estimated as colony diameter.

Conidiation was examined on plates containing 25 ml of solid GMM and GMM, supplemented with 200 mM or 400 mM KCl that were topagar inoculated with 5 ml of GMM, GMM supplemented with 200 mM or 400 mM KCl containing 5 x10⁶ spores from the strains ∆*sltA*, *veA1* (HHF27B) or ∆*sltA*, *veA*’ (RSS1.6P), or from the corresponding control strains. The cultures were incubated for 5 days at 37°C. The experiment was carried out with three replicates. After 5 days, 8-mm-diameter cores were obtained from each spread plate and homogenized in water. Conidial production was quantified using a hemocytometer. For evaluation of cleistothecial production, wrapped top-agar inoculated cultures were
Table 2. List of primers used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’–3’)</th>
</tr>
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<tbody>
<tr>
<td>actin-F</td>
<td>ATGGAGAAGGGAGATGTGGTTCTGCTCGTTATCGACAATGGTCC</td>
</tr>
<tr>
<td>actin-R</td>
<td>GTGAAGGAACTAGGGACAGGGACGAGGG</td>
</tr>
<tr>
<td>nsdD-F</td>
<td>CATCTCGACGGCAGCCATTACAGAGGGGACACAACATAC</td>
</tr>
<tr>
<td>nsdD-R</td>
<td>TTGGCAAGACGACAAAGCTGTTACAGCAGTCCTGGA</td>
</tr>
<tr>
<td>steA-F</td>
<td>TCCAGCAATTGGAGACGGTGAATGCTCGTCAC</td>
</tr>
<tr>
<td>steA-R</td>
<td>GAAAGTGAGGGCGCAATAGGAGCTCTGCGGTGAA</td>
</tr>
<tr>
<td>sltA-F</td>
<td>AGCTGCGTGTGACGTTAGGTGTTGTTGTTGTTG</td>
</tr>
<tr>
<td>sltA-R</td>
<td>CGGAAAGAAATGTCTGACAGGAGGACATACCTGGA</td>
</tr>
<tr>
<td>aflF-F</td>
<td>ATGGAGCACCCAGCGATCAGCCAG</td>
</tr>
<tr>
<td>aflF-R</td>
<td>TTGGTGTATGCTGCTGTCTTTGCTGCTCA</td>
</tr>
<tr>
<td>sctu-F</td>
<td>GTCTGGATGGAAGAAGCTGCTGTAACGGGG</td>
</tr>
<tr>
<td>sctu-R</td>
<td>CATGCCCGAAGCAGAACAAATCCTCCGGTTAG</td>
</tr>
<tr>
<td>sltA39</td>
<td>GAGGAAACCATCAAGGAGGG</td>
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<tr>
<td>sltA47</td>
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<td>sltA13</td>
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</tr>
<tr>
<td>sltA_F</td>
<td>CGTCTGCCGCTCTCCTCCTCACC</td>
</tr>
<tr>
<td>sltA_R</td>
<td>GGTACAATCCCGGTACGTG</td>
</tr>
</tbody>
</table>

incubated for 10 days at 37°C, when 16-mm-diameter cores were obtained from each culture and were frozen with 70% ethanol to enhance visualization of cleistothecia.

Alternatively, 10⁷ spores/mL of the ∆sltA, veA+ (RSS1.6P) strain, along with its control, were inoculated into flasks containing either GMM, GMM supplemented with 200 mM KCl or GMM supplemented with 10 mM CaCl₂. Cultures were grown in a shaker incubator for 24 h at 37°C at 250 rpm. Then, approximately 2 grams of mycelia were transferred onto each of the corresponding solid plates containing GMM or GMM supplemented with salts (KCl or CaCl₂). The plates were then incubated for additional 24 and 48 h. Then, 8-mm-diameter cores were obtained from each plate and homogenized. Conidial and Hüle cell production were evaluated using a hemocytometer. The experiment was carried out with three replicates.

Mycotoxin analysis
Three cores (16-mm diameter) were collected from each replicate of the cultures described above (both spread plating and shift culture) and extracted with CHCl₃. The extracts were analyzed by thin-layer chromatography (TLC). Benzene; glacial acetic acid (95:5 [vol/vol]) or alternatively toluene: ethyl acetate: acetic acid (80:10:10 [vol/vol]) were used as solvent systems. The silica gel TLC plates were then sprayed with aluminum chloride (12.5% concentration in ethanol 95%) and baked for 10 min at 80°C prior to viewing to intensify ST fluorescence upon exposure to long-wave (375 nm) UV light. ST purchased from Sigma was used as a standard. Densitometry of the TLC images was carried out using Scion Image 4.03 software.

Gene expression analysis
Expression analysis of developmental genes was carried out using mycelia harvested from shift (liquid to solid) cultures of ∆sltA (RSS1.6P) and its veA+ control strain (FGSC4) at 24 and 48 hours after the shift. Samples were frozen and stored at -80°C. Expression analysis of mycotoxin genes was performed from mycelia harvested from liquid shaken cultures. In both cases, samples were lyophilized, followed by RNA isolation using Trizol® reagent according to the manufacturer recommendations. Gene expression was analyzed by quantitative reverse transcription-PCR (qRT-PCR). Five micrograms of total RNA were treated with RQ1 RNase-Free DNase (Promega) and reverse transcribed with Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega) to synthesize cDNA. Applied Biosystems 7000 Real-Time PCR System was used for qRT-PCR and SYBR green dye was used for fluorescence detection. The primer pairs used for qRT-PCR are listed in Table 2. The data for each gene were normalized to the A. nidulans housekeeping gene, actin-F and the relative expression levels were calculated using the 2^−ΔΔCT method described previously [29].

Expression profile of sltA during vegetative growth, as well as during asexual and sexual-developmental phases was examined in a wild-type strain (veA+, WIM126) grown for 12, 18 and 24 h in liquid GMM to obtain vegetative mycelial growth. For induction of asexual or sexual development, vegetative mycelia were collected from liquid GMM, placed on top of solid GMM and incubated in the light (conducive to asexual development) or in the dark (conducive to sexual development). Samples were harvested at 6, 12, 24 and 48 h after the shift. Total RNA was extracted with Trizol® and analyzed by Northern blot. Extracted RNAs were probed with a radioactive-labeled sltA coding region PCR amplified from A. nidulans genomic DNA with primers sltA39 and sltA13 (Table 2).

In silico analysis of the promoter region
In order to identify putative SltA transcription factor binding sites, approximately 3.5 kb sequences upstream of the coding region of nsdD, brlA, steA and aflF were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/) and the Aspergillus Comparative Database (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html) for A. nidulans. The putative SltA binding sites were identified by comparison with the consensus sequences (5’ AGGCA 3’) previously described [4,5].

Results

sltA is important for growth and asexual development in A. nidulans
Classically Käfer’s GMM containing sodium nitrate as main nitrogen source has been used to study regulation of mycotoxin production and many aspects of development in Aspergillus nidulans [16,25,26,30–32]. For this reason this medium was used to investigate the role of SltA transcription factor in these processes. In previous studies carried out by Spielvogel et al., 2008 [4] on sltA mutant in veA1 genetic background only a slight reduction of growth was observed on Cove’s minimal medium with ammonium tartrate as nitrogen source (not conducive to mycotoxin production in A. nidulans) but strong
sensitivity was detected to elevated concentrations of mono and divalent cations (Figure S1) with the exception of calcium [4]. In our current study, the A. nidulans sltA deletion strains and their control strains were exposed to different salt concentrations in GMM in order to elucidate the possible role of sltA on fungal growth and asexual development in relationship with the cation concentration in the environment, and specifically we selected K⁺ for this purpose. The growth of ∆sltA strains was significantly reduced in comparison with the control strain, and this reduction was further pronounced in the presence of K⁺ (Figures 1 and S1, S2), particularly in the presence of 400 mM KCl (Figure 1A and 1C Figures S2A and S2C), indicating that sltA is important for fungal growth. The reduction of growth observed on GMM was independent of the type of veA allele. Interestingly, the addition of 10 mM CaCl₂ to GMM resulted in a significant increase in the fungal colony growth in the sltA deletion mutant strain (Figure 2).

In order to prevent the possible effect of growth reduction on the evaluation of the role of SltA on morphogenesis, the strains were top-agar inoculated, or alternatively, a liquid-to-solid mycelial transfer was implemented using the same amount of biomass for all strains. A decrease in the production of conidia was observed in the absence of sltA (in both, veA1 and veA⁰ genetic backgrounds) particularly in the presence of 200 mM KCl (Figures 1D and S2D) and even more as the concentration was increased to 400 mM (Figures 1E and S2E).

sltA is required for normal brlA expression

Expression of brlA, a master gene encoding a transcription factor necessary for asexual development in A. nidulans [11], was analyzed in ∆sltA, veA⁰ (RSS1.6P) and its control strain (FGSC4). In order to obtain quality RNA from mycelia, the strains were grown in liquid shaken culture for about 24 hours and then equal amounts of mycelia were transferred onto solid plates and incubated for an additional 24 and 48 hours to allow asexual development. Expression analysis of brlA was dependent on the cation content for the wild-type strain. At 24 hrs brlA mRNA levels were increased more than twice when K⁺ was added, and more than 7 times when Ca²⁺ was supplemented. This positive effect on brlA transcription resulted in an increase in condiation in both K⁺ and Ca²⁺ supplemented medium (Figure 3A and 3B). Asexual development was greatly reduced in the sltA deletion mutant grown with or without K⁺ at 24 hour time point, accompanied by a decrease in brlA expression, as compared to the wild type (Figure 3A and 3B). Addition of 10 mM CaCl₂ to GMM resulted in a significant recovery of brlA expression in the ∆sltA strain (Figure 3A), with an increase in condiation (Figure 3B). This positive effect of calcium on brlA expression in both wild type and null sltA decreased over time (Figure 3A). This transitory effect was also observed at 24 h for the wild-type strain when K⁺ was supplemented to the medium.

sltA is necessary for sexual development

Sexual development was also altered in the ∆sltA, veA⁰ (RSS1.6P) strain. Deletion of sltA resulted in a strain unable to produce cleistothecia (Figure 4). In contrast to the positive effect of addition of K⁺ on asexual development, production of mature cleistothecia was negatively affected by the addition of this cation in the wild type (Figure 4). However, calcium promoted both asexual and sexual development, increasing cleistothecia formation in both sltA mutant and wild-type strains (Figure 4).

Deletion of sltA affected early stages of sexual development. A reduction in Hülle cell production was observed in the sltA deletion mutant (RSS1.6P) compared to the control (FGSC4) 24 and 48 hours after the shift from liquid to solid GMM (Figure 5A). Addition of K⁺ had no positive effect but calcium partially recovered Hülle cell production in the mutant background (Figure 5A).

Normal expression of nsdD and steA requires a sltA wild-type allele

We examined whether this defect in the first stages of sexual development was caused by changes in the expression of nsdD and steA genes, necessary for sexual development in A. nidulans [33,34]. nsdD expression was reduced in the ∆sltA strain (RSS1.6P) as compared to the corresponding control strain (Figure 5B). Supplementation with K⁺ resulted in an additional delay in nsdD expression in the sltA deletion mutant. Furthermore, steA was also reduced in the ∆sltA strain as compared to the wild type, at both time points analyzed (Figure 5C). Addition of 10 mM Ca²⁺ resulted in a notable increase in nsdD and steA transcript levels, particularly in the sltA mutant strain, at both time points, 24 and 48 hours respectively (Figure 5B and 5C).

Expression of sltA during different developmental stages

Due to the observed effect of sltA deletion on the expression of developmental genes regulating both asexual and sexual morphological differentiation, we also analyzed the expression of sltA in the wild type during vegetative growth and also during different developmental stages. Our Northern blot analysis indicated that sltA was expressed during vegetative growth as well as during asexual and sexual differentiation at each time point analyzed (Figure 6), with the greatest sltA expression during vegetative growth.

Role of sltA in A. nidulans ST production

Mycotoxin analysis indicated that the production of ST is drastically reduced in the sltA deletion strains (RSS1.6P and HHF27B) compared to the control strains (FGSC4 and HHF27A) in the veA⁰ or veA¹ genetic background, respectively (Figure 7 and Figure S3), particularly in the presence of 200 mM or 400 mM KCl. Interestingly, ST biosynthesis was partially recovered in the sltA deletion strain cultures containing GMM supplemented with 10 mM CaCl₂ (Figure 7).

Expression of ST genes is sltA-dependent

Expression of aflR, encoding a transcription factor necessary for the activation of ST gene cluster, and stcU, a structural gene in the cluster commonly used as indicator of ST cluster activation, were slightly reduced in the sltA deletion strain (RSS1.6P) compared to the wild-type control (Figure 8A and
Figure 1. SltA is necessary for normal growth and conidiation. A) Wild-type (FGSC4) and ΔsltA, veA+ (RSS1.6P) point-inoculated cultures containing GMM, or GMM supplemented with 200 mM or 400 mM KCl were incubated at 37°C for 5 days. B) & C) Measurement of the radial colony growth. D) & E) Quantification of conidial production from top-agar inoculated cultures (5 x 10⁶ spores/plate). Values are means of three replicates. The error bar indicates standard error.

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Potassium acted positively on the expression of these mycotoxin genes in the wild type strain, whereas had a strong negative effect in the null sltA mutant (Figure 8A and 8B). Mycotoxin analysis performed under the same experimental conditions also revealed a drastic decrease in ST production in ∆sltA with respect to the control (Figure 8C, 8D and 8E). Addition of calcium resulted in increased levels of aflR and stcU expression as well as ST production in ∆sltA.

Complementation of ∆sltA with a sltA wild-type allele remediated the defects in growth, morphogenesis and mycotoxin production

When a genomic wild-type sltA copy was transformed in ∆sltA, veA+ (RSS1.6P) the resulting complementation strain recovered wild-type phenotype (FGSC4 phenotype) for growth, asexual and sexual development and production of ST toxin (Figure S4), in addition to tolerance of high concentrations of divalent or monovalent cations (data not shown). ∆sltA, veA1 (HHF27B) was also complemented for loss of sltA function using a similar approach. All mutant phenotypes described to be associated with the absence of sltA in this work and in previous work [4] were suppressed (data not shown).

Promoter analysis

Analysis of the promoter region, consisting of about 3.5 kb upstream of the developmental genes brlA, nsdD and steA and mycotoxin, sterigmatocystin regulatory gene aflR revealed the presence of several putative SltA (5'-AGGCA- 3') binding sites: brlA (five), nsdD (one), steA (three) and aflR (five) (Figure S5). Analysis of the complementary strand of each of these promoter regions also revealed additional putative 5'-AGGCA-3' (SltA) target sites: brlA (three), nsdD (five), steA (four) and aflR (three) (Figure S5).

Discussion

In this study we have described the role of SltA, the transcription factor mediating cation stress response, on growth, morphological development (asexual and sexual) and ST production in the model fungus A. nidulans. SltA has a role in preventing the toxicity of cations such as Na+ and K+ but not by Ca2+; these studies were carried out in strains with veA1 allele [2–4,10], a mutant allele commonly present in many strains used in A. nidulans research laboratories which affects fungal development. In this study SltA function was characterized in veA+ (wild type) as well as in veA1 genetic background.

Our results have revealed a reduction in colony growth and conidial production in the A. nidulans sltA deletion mutant, in both veA1 or veA+ genetic backgrounds, indicating that sltA is important in fungal growth independently of the type of veA allele. The supplementation of potassium resulted in a minimal difference in colony growth of the sltA deletion mutant, already very reduced even in the absence of this excess of cation in nitrate-GMM. This medium has been used for decades in genetic studies of both development and mycotoxin production [16,25,26,30–32]. Previous work by Spielvogel et al., 2008 [4] on sltA mutant in veA1 genetic background presented slightly reduced colony growth when grown on Cove’s minimal medium with ammonium tartrate as nitrogen source. However this medium is not suitable to mycotoxin production in A. nidulans [35]. These differences in medium composition account for the variations observed in the growth of the sltA, veA1 mutant in
both studies ([4] and Figure S1). It is possible that a connection between nitrogen metabolism and the role of SltA could exist in A. nidulans.

Our study also showed that the reduction in conidiation was accompanied by a reduction in the expression of brlA, a key regulator of asexual development [11], in the \( \Delta \text{sltA} \) as compared to the wild type. The presence of \( K^+ \) had an opposite effect in the wild type versus the \( \Delta \text{sltA} \) mutant. Potassium elevated \( \text{brlA} \) expression and conidiation in the wild type while it caused a strong decrease in the absence of SltA. This indicates that SltA is not only necessary for normal colony growth but also required for wild-type expression of \( \text{brlA} \) and concomitant conidiation in A. nidulans, particularly under potassium stress conditions.

Promoter analysis of \( \text{brlA} \) showed the presence of eight 5'-AGGCA-3' consensus SltA binding sites (Figure S5). These are distributed within the 2.3 kb region upstream of the \( \text{brlA} \) translation start site. Interestingly, sites located at -2275 and -2300, the furthest upstream sites, are in a region that also contains experimentally demonstrated FlbB and FlbD binding sites [36]. The abundance of SltA binding sites together with the fact that this region is subjected to regulation by other DNA binding proteins strongly suggests these putative SltA sites are functional.

In this study we showed that \( \text{sltA} \) transcripts are present during asexual and sexual stages, and demonstrated that sexual development is also impaired in the absence of \( \text{sltA} \), coinciding with lower expression of \( \text{nsdD} \) and \( \text{steA} \), known to be necessary for sexual development [33,34]. \( \text{nsdD} \) and \( \text{steA} \) promoter regions also presented putative SltA binding sites. Both sexual and asexual developmental programs were partially recovered when \( \Delta \text{sltA} \) cultures were supplemented with calcium, as well as an overall recovery of fungal growth. In Penicillium urticae, calcium was shown to increase conidiation [37]. Addition of low amounts of calcium to the medium, also led to increased accumulation of \( \text{brlA} \) transcripts in the \( \Delta \text{sltA} \) deletion strain. Similarly, partial recovery of Hülle cells, nursing cells for cleistothecial formation [13] coincided with a recovery of \( \text{nsdD} \) and \( \text{steA} \) transcript levels, which are abnormally low in \( \Delta \text{sltA} \) cultures.

Previous reports showed that calcium alleviates a number of defects caused by the null \( \text{sltA} \) in early growth stages. For instance, \( \text{sltA} \) strains present altered number, size and distribution of vacuoles, and calcium remediates this phenotype [10]. Abnormal expression of calcium vacuolar ATPase coding genes, \( \text{pmcA} \) and \( \text{pmcB} \), were observed in \( \Delta \text{sltA} \) background and such phenotype was associated with a cytoplasmic Ca\(^{2+}\) depletion. This effect of calcium auxotrophy was caused by the combination of \( \Delta \text{sltA} \) with a null allele of \( \text{halA} \), a kinase
involved in cation stress signaling [10,38]. Findon et al. [10] also showed the link between alkaline cation uptake systems and calcium through the isolation of suppressors of "calcium auxotrophy" phenotype, of which several mutations were isolated in a Na\(^{+}/H^{+}\) transporter, NhaA, and in a K\(^{+}\) transporter, TrkB [10]. Additionally a role of SltA in intracellular traffic of endomembranes has been proposed as a result of isolation of loss-of-function mutations in \(\text{sltA}\) as extragenic suppressors of the lethal phenotype caused by null alleles of certain vacuolar-protein-sorting (vps) genes. An interpretation of this genetic effect of \(\text{sltA}\) mutations was an altered plasma-membrane trafficking of small vesicles derived from extreme mutations in these \(\text{vps}\) genes [39]. It is possible that the remediation of colonial growth, asexual and sexual development defects observed in the \(\text{sltA}\) deletion mutant supplemented with calcium could be the result of a restoration of intracellular membrane-based trafficking and/or signaling systems. Previously, it was also shown that high gradient of Ca\(^{2+}\) is necessary for tip hyphal extension in filamentous fungi [40]. Decreasing the Ca\(^{2+}\) influx leads to reduction of hyphal extension and increased branching, while high extracellular calcium inhibits branching and induces hyphal elongation [41]. Calmodulin is an essential signaling element that senses intracellular calcium and accumulates at the hyphal tip in \(A. \text{nidulans}\) [42]. To date a relationship between CaM and SltA has not been investigated but the possible effect of SltA on the regulation of free cytosolic calcium might alter the signaling cascade depending on calmodulin-Ca\(^{2+}\) complex, affecting, among other cellular processes, the calcium gradient that promotes hyphal tip elongation.

Although a positive effect of calcium in conidiation has been also reported in \(\text{Penicillium}\) species [43,44], this is, to our knowledge, the first report showing this Ca\(^{2+}\)-dependent conidiation increase in \(A. \text{nidulans}\). In our studies this increase in asexual development was accompanied by a remarkable increase in \(\text{brlA}\) expression. Calcium signaling and \(\text{brlA}\) expression has been connected through studies on the transcription factor \(\text{CrzA}\) in \(A. \text{fumigatus}\), and the activity of the protein phosphatase calcineurin which has been reported to have a role in conidiation and sexual development [45].

A regulatory link between development and secondary metabolism has been experimentally demonstrated in filamentous fungi, including the model fungus \(A. \text{nidulans}\) [14,16,17,46]. For this reason we investigated the role of \(\text{sltA}\) in mycotoxin production. Under standard culture conditions \(\Delta\text{sltA}\) showed a decrease in ST biosynthesis compared to the wild type. This decrease was even more notable under potassium stress either in \(\text{veA}^+\) or \(\text{veA}^+\) genetic backgrounds, coinciding with delayed expression of \(\text{aflR}\) and \(\text{stcU}\). This indicates that \(\text{sltA}\) influences ST by controlling \(\text{aflR}\) and \(\text{stcU}\). This indicates that \(\text{sltA}\) influences ST by controlling \(\text{aflR}\) and consequently the activation of the ST gene cluster. Putative SltA binding sites are also present in the \(\text{aflR}\) promoter region. Addition of calcium not only partially rescued the morphological defects in the \(\Delta\text{sltA}\) mutant, but also resulted in a recovery in the levels of \(\text{aflR}\) and \(\text{stcU}\) expression levels. Additionally to the described effect of \(\text{sltA}\) deletion on the expression of ST genes, possible alterations in vesicle trafficking [39] could also contribute to the observed decrease in mycotoxin biosynthesis [48].

Figure 4. Effect of \(\text{sltA}\) deletion on \(A. \text{nidulans}\) cleistothecial production. Micrographs of fruiting bodies (cleistothecia) in \(\Delta\text{sltA}, \text{veA}^+\) (RSS1.6P) and wild type (FGSC4). Strains were top-inoculated with 5 \(\times 10^6\) conidia per plate on GMM, GMM supplemented with KCl (200 mM or 400 mM), or GMM supplemented with 10 mM CaCl\(_2\), and incubated at 37°C for 10 days. Plates were wrapped to promote sexual development. Cultures were sprayed with 70% ethanol to facilitate the visualization of cleistothecia. Arrows indicate cleistothecia in the \(\Delta\text{sltA}, \text{veA}^+\) culture. Magnification: x50.

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Developmental defects and a reduction of ST production in ∆sltA supports the role of SltA in normal growth, conidiation, sexual development and mycotoxin production in *A. nidulans*, suggesting that maintaining homeostasis and proper response to changes in the concentration of cations are necessary to allow several cellular functions including morphogenesis and secondary metabolism. These results contribute to a better understanding of the roles associated with cation regulation and signaling mechanisms in filamentous fungi, particularly in the genus *Aspergillus*.

**Figure 5.** SltA is required for normal Hülle cell production and regulates *nsdD* and *steA* expression. A) Quantification of Hülle cells produced by wild-type (FGSC4) and ∆sltA, veA+ strains (RSS1.6P). The strains were first grown in liquid shaken cultures for 24 hours at 250 rpm. Then equal amounts of mycelia were homogeneously spread onto solid GMM, GMM supplemented with 200 mM KCl, or GMM supplemented with 10 mM CaCl₂ (time point - 0 h) and further incubated at 37°C. B) and C) Expression levels of *nsdD* and *steA*, respectively, analyzed by qRT-PCR. Total RNA was extracted using Trizol®, from mycelial samples collected at 24 and 48 hours after the shift. The values were normalized to the wild-type (grown on GMM -24 h) levels considered as 100. The error bar represents standard error. Values are the means of three replicates.

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Supporting Information

Figure S1. Effect of medium on veA1::sltA and veA+::sltA colony growth. ΔsltAveA1 (HHF27B), ΔsltAveA+ (RSS1.6P) and corresponding control strains (HHF27A and FGSC4 respectively) were point-inoculated on plates containing GMM as described by Käfer [28], or GMM supplemented with 200 mM, containing sodium nitrate as nitrogen source (A, B), or on minimum medium as described by Cove [10,47], or Cove medium plus 200 mM, containing ammonium tartrate as nitrogen source (C, D). Cultures were incubated at 37°C for 5 days. (TIF)
Figure S2. SltA is necessary for normal growth and conidiation also in a veA1 genetic background. A) Control strain (HHF27A) and ΔsltA, veA1 (HHF27B) point-inoculated cultures containing GMM, or GMM supplemented with 200 mM KCl or 10 mM CaCl₂ were incubated at 37°C for 5 days. B) & C) Measurement of the radial colony growth. D) & E) Quantification of conidial production from top-agar inoculated cultures (5 x 10⁵ spores/plate). Values are means of three replicates. The error bar indicates standard error.

Figure S3. Effect of sltA deletion on ST biosynthesis was similar in both veA+ and veA1 genetic backgrounds. A) & C) TLC analysis of ST produced in wild-type (FGSC4) and ΔsltA, veA+ (RSS1.6P) cultures under the same experimental conditions. D) & E) Densitometry displaying the relative intensity of ST bands. Values are the means of two replicates. The error bar represents standard error.

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400 mM). Strains were top-inoculated with 5 x 10⁶ conidia per plate and incubated at 37°C for 5 days. ST was extracted and analyzed as described in the experimental procedure section. B) & D) densitometry displaying the intensity of the ST bands in A and C respectively. E) & G) TLC analysis of ST produced by the HHF27B control and ΔsltA, veA strains on GMM or GMM supplemented with KCl (200 and 400 mM). The experiment was carried out as above. F) and H) densitometry displaying the intensity of the ST bands in E and G respectively. Densitometries were carried out using the Scion Image 4.03 software. Values are the means of three replicates. The error bar represents standard error.

(TIF)

Figure S4. Complementation of ΔsltA with asltA wild-type allele rescues wild-type phenotype.
A) For reconstitution of null sltA into a wild-type sltA locus, a PCR fragment amplified using primer pair sltA_F and sltA_R containing the wild-type sltA genomic sequence between coordinates 2396219 and 2400452 of chromosome VI (http://www.aspgd.org/) was used for transformation of ΔsltA, veA* prototroph (RSS1.6P). Positive transformants recovering SttA activity were selected onto glucose minimal medium containing 1M sucrose as osmotic stabilizer and 0.3 M LiCl. Presence of a wild-type genomic copy of sltA was verified by PCR techniques. Agarose electrophoresis of PCR products using oligonucleotides sltA47 and sltA39. Lanes contain PCR products using as template genomic DNAs from RSS1.6P (lane 1), FGSC4 (lane 2), HHF27B (lane 3), reconstituted sltA transformants in RSS1.6P (lanes 4–9), a reconstituted sltA transformant in HHF27B (lane 10). Lanes A and B are control PCRs for negative amplification of sltA locus using HHF27A genomic DNA. Absence of mutations in the coding region of sltA was verified by sequencing. Mw is DNA molecular maker VII (Roche). Complementation of ΔsltA, veA* rescues wild-type conidiation and cleistothecial production in A. nidulans (B), as well as ST biosynthesis (C). Plates in (B) were top-agar inoculated with 5 x 10⁶ spores of wild type (FGSC4), ΔsltA, veA* + (RSS1.6P), and complementation strain (RSS1.6P-com) on GMM or GMM plus 200 mM KCl, and incubated for 5 days. ST shown in (C) was extracted from liquid shaken cultures grown for 4 days.

(TIF)

Figure S5. In-silico analysis of putative SltA transcription factor binding sites in the promoter regions. A) brlA B) nsdC D) steA and D) αfR. Numbers indicate the positions of the putative SltA binding site upstream to the ATG start. Green solid lines (5’–3’) indicate the putative SltA binding sites identified in the 3.5 kb region upstream of the above mentioned genes, while blue solid lines indicate the SltA binding sites on the complementary strand (3’–5’) of the promoter region. Coordinates at both ends of the promoter regions are with respect to the position in the contig. Yellow boxes indicate CDS and white boxes indicate exons of 5’ and 3’ UTRs present in the genomic regions under analyses. In the case of brlA, ATG of brlA alpha transcript is shown but also the position of brlA beta initiation codon and first exon.

(TIF)

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Author Contributions

Conceived and designed the experiments: SS SD PH EAE AMC. Performed the experiments: SS SD PH. Analyzed the data: SS SD EAE AMC. Contributed reagents/materials/analysis tools: AMC. Wrote the manuscript: SS EAE AMC.

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