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Negative regulation of the vacuole-mediated resistance to K^+ stress by a novel C_2H_2 zinc finger transcription factor encoded by *aslA* in *Aspergillus nidulans*

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In fungi and plants, vacuoles function as a storage and sequestration vessel for a wide variety of ions and are responsible for cytosolic ion homeostasis and responses to ionic shock. In the filamentous fungus *Aspergillus nidulans*, however, little is known about the molecular genetic mechanisms of vacuolar biogenesis and function. In the present study, we analyzed the function of the *aslA* gene (AN5583) encoding a novel C_2H_2-type zinc finger transcription factor (TF) in relation to K^+ stress resistance, vacuolar morphology, and vacuolar transporters. The mutant lacking *aslA* showed increased mycelial growth and decreased branching at high K^+ concentrations. Deletion of *aslA* also caused elevated K^+ stress-inducible expression of the genes, *nhxA* (AN2288), *vnxA* (AN6986), and *vcxA* (AN0471), encoding putative endosomal and vacuolar cation/H^+ exchangers, as well as *cpyA* and *vpsA* genes encoding the proteins involved in vacuolar biogenesis. Interestingly, vacuolar fragmentation induced by K^+ stress was alleviated by *aslA* deletion, resulting in persistence of unfragmented vacuoles. In the presence of bafilomycin, an inhibitor of vacuolar H^+-ATPase, the mutant phenotype was suppressed in terms of growth rates and vacuolar morphology. These results together suggest that the C_2H_2-type zinc finger TF AslA attenuates the K^+ stress-inducible expression of the genes encoding the ion pumps involved in vacuolar sequestration of K^+ ions powered by vacuolar H^+-ATPase, as well as the proteins that function in vacuolar biogenesis.

Keywords: K^+ resistance, C_2H_2 zinc finger transcription factor, vacuole, *aslA*, *Aspergillus nidulans*

Introduction

Fungi grow in a wide range of environmental niches and are exposed to various forms of stresses, such as osmotic stress, oxidative stress, starvation stress, heat stress, and salinity stress. Thus, fungi need to respond adequately to environmental stresses to enable their cells to endure the adverse environment. When exposed to high concentrations of salts, fungi are subjected to both osmotic stress and ion toxicity. In response to hyperosmotic stress, compatible solutes, mainly glycerol and trehalose, are accumulated to compensate for the difference between the extra- and intra-cellular water potential (Hohmann, 2002; Hohmann *et al.*, 2007). In addition, fungi are equipped with several mechanisms to keep low levels of salt in the cytoplasm to alleviate the adverse effects of salt stress on growth and development. One such mechanism is vacuolar sequestration of potentially toxic ions, like sodium and chloride, or other compounds involved in osmoregulation. As a storage and sequestration vessel for a wide variety of ions, the vacuole is the main organelle responsible for cytosolic ion homeostasis and response to ionic shock (Li and Kane, 2009).

Vacuoles are subjected to morphological changes in response to environmental stresses. Exposure of *Saccharomyces cerevisiae* cells to hypertonic stress leads to fragmentation of the vacuoles into numerous small vacuolar vesicles, which probably allow the cells to restore the osmotic balance by readapting the surface-to-volume ratio of the compartment after the osmotic loss of water (Zieger and Mayer, 2012). The dynamin-like GTPase, Vps1p, which is localized to vacuoles, is implicated in the regulation of both vacuolar fission and fusion (Peters *et al.*, 2004). Vacuolar fission also requires the proton pump activity of vacuolar H^+-ATPase (V-ATPase) (Baars *et al.*, 2007) and the lipid phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$) (Gary *et al.*, 1998; Bonangelino *et al.*, 2002). PI(3,5)P2 is synthesized from phosphatidylinositol by two consecutive phosphorylations catalyzed by the phosphoinositide 3-kinase (PI 3-kinase), Vps34, and the phosphatidylinositol 3-phosphate (PI(3)P) 5-kinase, Fab1 (Cooke *et al.*, 1998; Gary *et al.*, 1998; Efe *et al.*, 2005). Fab1p kinase activity is regulated by three other proteins: Vac7, Vac14, and Fig4 (Bonangelino *et al.*, 2002; Dove *et al.*, 2002; Gary *et al.*, 2002).

S. cerevisiae cells are equipped with several vacuolar transporters that mediate ion homeostasis by regulating ion transport between the vacuole and cytoplasm. The vacuolar monovalent cation/H^+ antiporter Vnx1 is a low affinity $Na^+(K^+)/H^+$ exchanger with a higher affinity for Na^+ than for K^+ (Cagnac *et al.*, 2007). Despite the homology with other members of the calcium exchanger (CAX) family of transporters, Vnx1 is unable to mediate Ca^{2+} transport. Vnx1 plays roles in the regulation of ion homeostasis and cellular pH in a V-ATPase-dependent manner (Cagnac *et al.*, 2007). Vcx1, a vacuolar

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(A)

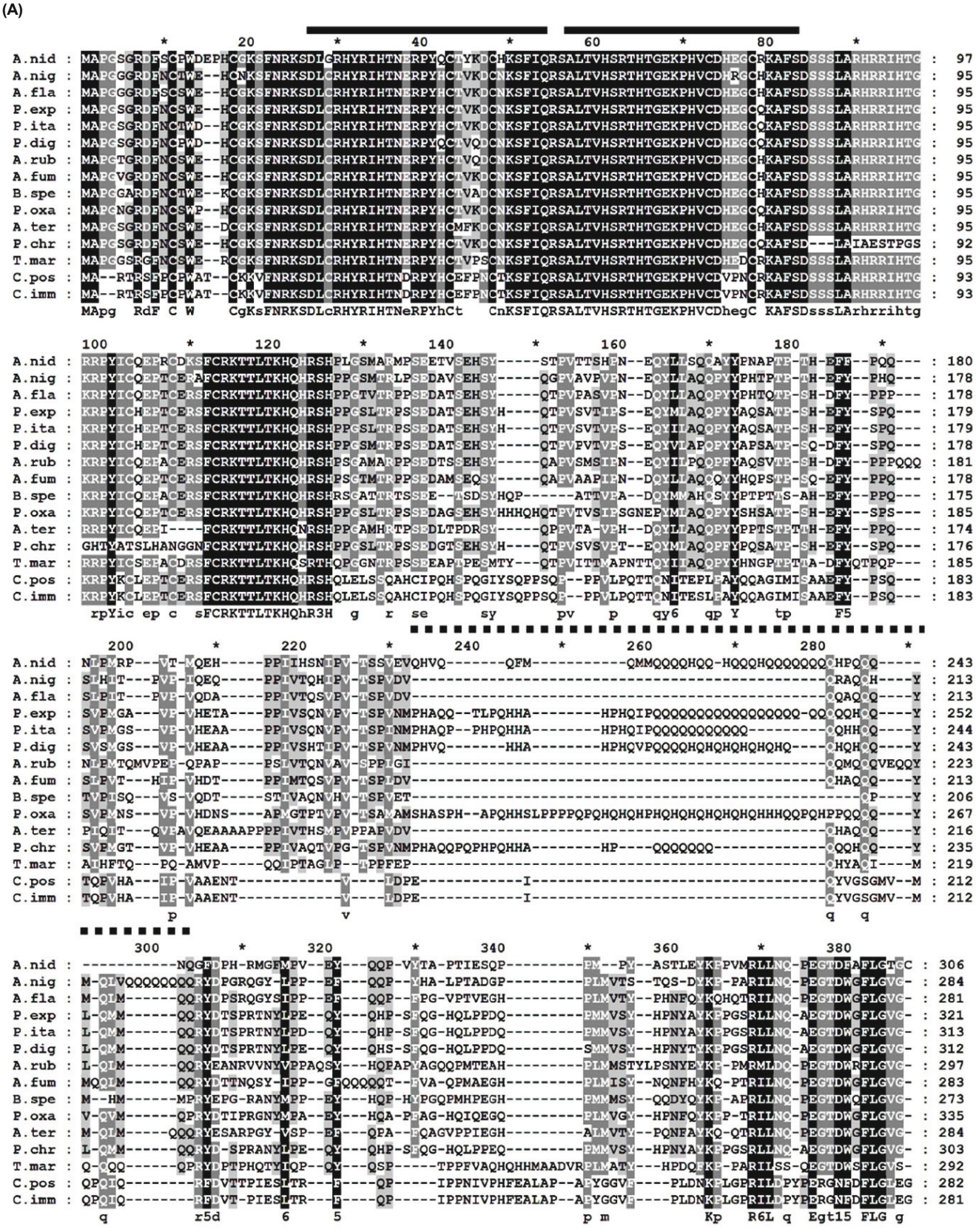

Fig. 1. Continued

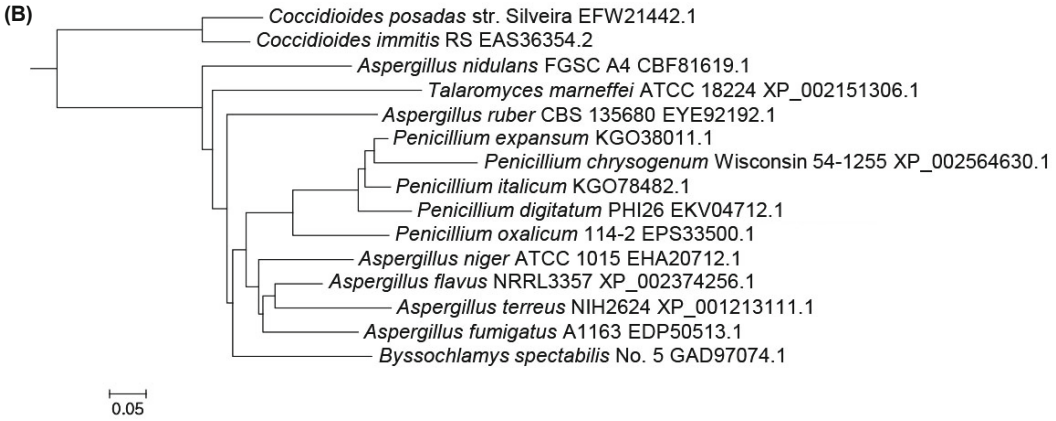

Fig. 1. Analysis of the amino acid sequence of *A. nidulans* AslA. (A) Multiple alignment of the deduced amino acid sequences of AslA and its orthologues in different fungal species created Genedoc software (version 2.6.003; http://www.psc.edu/biomed/genedoc). Continuous dark line, C_2H_2 zinc finger domain; broken line, glutamine rich domain. Abbreviations: A.nid, *A. nidulans*; A.nig, *Aspergillus niger*; A.fla, *Aspergillus flavus*; A.fum, *Aspergillus fumigatus*; A.rub, *Aspergillus ruber*; A.ter, *Aspergillus terreus*; P.chr, *Penicillium chrysogenum*; P.exp, *Penicillium exspansum*; P.ita, *Penicillium italicum*; P.dig, *Penicillium digitatum*; P.oxa, *Penicillium oxalicum*; T.mar, *Talaromyces marneffei*; B.spe, *Byssochlamys spectabilis*; C.pos, *Coccidioides posadas*; C.imm, *Coccidioides immitis*. (B) Phylogenetic and molecular evolutionary analyses of AslA and its orthologues were conducted using MEGA software, version 3.1 (neighbor- joining method, with a bootstrap of 50,000 replicates and amino p-distance substitution model).

Ca^{2+}/H^+ antiporter, has been originally identified to be one of the proteins that sequesters calcium into the vacuole, thus contributing to the maintenance of Ca^{2+} homeostasis (Cunningham and Fink, 1996). Subsequently, it has been shown that Vcx1 also functions as a K^+/H^+ antiporter along with Vnx1, thereby contributing to sequestration of cytosolic K^+ into vacuoles (Cagnac *et al.*, 2010). Nhx1, the first discovered intracellular antiporter of *S. cerevisiae* (Nass *et al.*, 1997), contributes to the sequestration of toxic Na^+ and Li^+ ions, as well as surplus K^+, in the endosomes and vacuoles (Quintero *et al.*, 2000; Brett *et al.*, 2005) and is involved in the early stage of cell adaptation to hyperosmotic shock (Nass and Rao, 1999). Unlike Vnx1 and Vcx1, Nhx1 does not perform direct transport of cations from the cytosol to the vacuole through the vacuolar membrane, but instead contributes to vacuolar accumulation of cations by fusion of cation-loaded vesicles trafficked from the prevacuolar compartment (PVC) to the vacuole or by controlling the trafficking to the vacuole of transporters involved in alkali cation homeostasis (Cagnac *et al.*, 2010).

Despite the extensive functional and structural characterization of the vacuolar/prevacuolar transporters for alkali metal ions in yeast, limited information on the regulation of their gene expression and activity has been obtained. Vcx1 is negatively regulated by Ca^{2+}/calmodulin-bound calcineurin at the transcriptional and, mainly, post-transcriptional levels (Cunningham and Fink, 1996). The negative regulation of Vcx1 by calcineurin is thought to be independent of the transcription factor Crz1, which regulates genes encoding ion pumps and cell wall biosynthesis enzymes (Matheos *et al.*, 1997; Stathopoulos and Cyert, 1997). In the filamentous fungus, *Aspergillus nidulans*, transcription of the *vcxA* gene encoding a putative vacuolar Ca^{2+}/H^+ exchanger, a homologue of *S. cerevisiae* Vcx1, is regulated negatively by SltA, which has no identifiable *S. cerevisiae* homologue, but positively by CrzA, a homologue of *S. cerevisiae* Crz1 (Spielvogel *et al.*, 2008). Although some notable work has been performed on calcineurin-related regulation of *vcxA* expression, the role and regulation of vacuolar transporters in relation to salt stress has been less investigated in filamentous fungi than in yeasts.

In the present study, we analyzed the function of the *aslA* gene (AN5583) encoding a novel C_2H_2-type zinc finger transcription factor (TF) in relation to K^+ stress resistance, vacuolar morphology, and vacuolar/prevacuolar transporters. Here, we report that *aslA* negatively contributes to K^+ stress tolerance and is responsible for vacuolar fragmentation in the presence of high concentrations of K^+ ion. In addition, we present evidence supporting that AslA attenuates the K^+ stress-inducible expression of several genes expected to be involved in vacuolar biogenesis and function.

Materials and Methods

Strains, media, cultivation, and transformation

The *A. nidulans* strains used in this study are listed in Table 1. Glucose minimal medium (MMG) consisted of 1% glucose, 0.6% $NaNO_3$, 0.052% KCl, 0.052% $MgSO_4{\cdot}7H_2O$, 0.152% KH_2PO_4, 0.1% (v/v) 1000× trace element solution (2.2% $ZnSO_4{\cdot}7H_2O$, 1.1% H_3BO_3, 0.5% $MnCl_2{\cdot}4H_2O$, 0.5% $FeSO_4{\cdot}7H_2O$, 0.16% $CoCl_2{\cdot}5H_2O$, 0.16% $CuSO_4{\cdot}5H_2O$, 0.11% $NaMoO_4{\cdot}$

Table 1. *A. nidulans* strains used in this study

Strains	Genotype	Source/References
FGSC4	*veA*$^+$	FGSC[a]
TJ1	*yA2*, *argB2*, *pyroA4*, *veA*$^+$	S. K. Chae
MCBA001	*yA2*, *pyroA4*, *veA*$^+$	This Study
MCBA101	*yA2*, *argB2*, *pyroA4*, *veA*$^+$, *ΔaslA::argB*	This Study
MCBA002	*yA2*, *argB2*, *veA*$^+$	This Study
MCBA201	*yA2*, *argB2*, *pyroA4*, *veA*$^+$, *ΔaslA::argB*, *aslA::pyroA*	This Study
MCBA301	*yA2*, *argB2*, *veA*$^+$, *alcA(p)::aslA::pyroA*	This Study

[a] Fungal Genetics Stock Center (USA)

$2H_2O$, 5% Na_2EDTA), and required supplements, such as 2.5 μM pyridoxine·HCl and/or 3.8 mM arginine (Lee *et al.*, 2004). Complete medium (CM) was prepared by adding 0.1% yeast extract, 0.1% casamino acid, 0.2% peptone, and 0.1% (v/v) 1,000× vitamin stock solution (0.1% *p*-aminobenzoic acid, 0.1% niacine, 0.1% pyridoxine·HCl, 0.1% riboflavin, 0.1% thiamine·HCl, 0.1% choline·HCl, and 2 ppm biotin) to MMG. For solid media, 2% agar was added to the liquid media. For overexpression of *aslA* mediated by the promoter of the *alcA* gene ($alcA_{(p)}$), threonine minimal medium (MMYT) identical to MMG, except that it contained 0.5% yeast extract and 100 mM threonine as a sole carbon source instead of 1% glucose, was used. The high salt media, MMGK and MMYTK, were prepared by supplementing MMG and MMYT with 1.2 M KCl. Transformation of *A. nidulans* and selection of transformants were performed as described previously (Lee *et al.*, 2004).

To prepare vegetative mycelia, conidia of the *A. nidulans* strains were inoculated into appropriate liquid media to a concentration of 1.0×10^6 conidia/ml and grown in a shaking culture at 120 rpm and 37°C. Balls of mycelia were then harvested by filtering the culture through a Miracloth filter (Calbiochem). To prepare K^+ stress-exposed and unexposed mycelia used for extraction of total RNA, the mycelia harvested from the culture grown for 15 h in liquid CM were shifted to liquid MMG and MMGK for 30 min, harvested, and frozen at -80°C until used. For microscopic observation of mycelial and vacuolar morphology, each *A. nidulans* strain was slide-cultured on a block of appropriate agar medium under a coverslip on a glass slide at 37°C for 1–2 days.

RNA preparation and qRT-PCR

Total RNAs were extracted from frozen mycelial samples by grinding in liquid nitrogen with a mortar and pestle followed by modified guanidine thiocyanate/CsCl density gradient ultracentrifugation (Sambrook and Russell, 2001). The quantity and quality of isolated RNA was determined by spectrophotometry. RNA integrity was determined by formaldehyde-agarose gel electrophoresis. For qRT-PCR, first strand cDNA was copied from a total RNA preparation using M-MLV reverse transcriptase (USB), 5× M-MLV RT reaction buffer (USB), RNase inhibitor (Amersham), and oligo dT primer (Amersham) according to the manufacturer's protocol. qRT-PCR was performed using a Bio-Rad CFX96 Real-Time PCR System (Bio-Rad) and a TOPreal™ qPCR 2X PreMIX Kit (Enzynomics), which were used in accordance with the manufacturers' instructions. Formation of PCR products was continuously monitored during the PCR reaction using CFX Manager software version 2.1 (Bio-Rad). For each strain/

Table 2. List of oligonucleotide primers used in this study

Primer	Sequence (5′→3′)	Gene (locus)
For deletion, complementation, and overexpression strains		
PaslA-5f	TTCGAGTCCGGTAAATTACC	*aslA*
PaslA-5r	ggtagatccaggcctaacacGGCGGATTAGAGGGCGCGGG[a]	*argB*, *aslA*
PaslA-3f	atgaggcctctaaactggtcGTGTGGTCTCTGGGTACGGA[a]	*argB*, *aslA*
PaslA-3r	TGAGTTAAAGGTGGTATTCC	*aslA*
PaslA-5fn	TCCGCTTGGTGTTTACCTTCG	*aslA*
PaslA-3rn	GAGCGACATTTCCGATTATC	*aslA*
PaslA-f	GGTACCTTCGAGTCCGGTAAATTACCAGT[b]	*aslA*
PaslA-r	AAGCTTTTAGCACCCAGTTCCAAGGAACG[c]	*aslA*
PaslA-0f	AAGCTTATGGCTCCTGGCAGCGGC[c]	*aslA*
PaslA-0r	AAGCTTGCACCCAGTTCCAAGGAACGC[c]	*aslA*
PargB-f	GACCAGTTTAGAGGCCTCAT	*argB*
PargB-r	GTGTTAGGCCTGGATCTACC	*argB*
For qRT-PCR		
PaslA-qf	CCTATGCCTCAACCCTTGAATACA	*aslA*
PaslA-qr	AAATCTGTTCCCTCTGGTTGATTTA	*aslA*
PacnA-qf	CTCCTACGTCGGTGATGAGGCAC	*acnA*
PacnA-qr	ATGTCATCCCAGTTCGTGACAACA	*acnA*
PnhxA-qf	ATGAATTTCTCCTTGTTTCTGCTT	*nhxA*
PnhxA-qr	AGATATAACTACCATTAGCTCGAA	*nhxA*
PvnxA-qf	GCGCAATGGGATATGATTACAG	*vnxA*
PvnxA-qr	TTTTACCTTCACCGTAGACATAAG	*vnxA*
PvcxA-qf	GTT AGTCTGCCATATATACGGCGA	*vcxA*
PvcxA-qr	GCA TCA ACGCATTGATAAGTCAAAG	*vcxA*
PcpyA-qf	ACTAGTCTTCATATCTTTCATTATTG	*cpyA*
PcpyA-qr	ATTTGAAATGACCCTGATTATGCAC	*cpyA*
PvpsA-qf	AAGTCATCA AGCTTCTTATTACCTC	*vpsA*
PvpsA-qr	TTCGGGACCATATCGATCATCGTG	*vpsA*

[a] Lowercase letters indicate *argB* sequence.
[b] Underlined letters indicate *Kpn*I restriction site.
[c] Underlined letters indicate *Hin*dIII restriction site.

culture condition, three independent biological replicates were performed. The primer sequences against the genes of interest and *acnA* (γ-actin gene; internal control) are presented in Table 2.

Expression levels of a target gene versus the housekeeping gene, *acnA*, were computed using the following formulae (Livak and Schmittgen, 2001):

Expression level of a target gene in a given sample = $2^{-(\Delta Ct)}$

where,

Ct = the cycle number at which logarithmic PCR plots of a gene cross a calculated threshold line, and

$\Delta Ct = Ct_{(target)} - Ct_{(acnA)}$

Relative expression of a target gene in a given sample = $2^{-\Delta(\Delta Ct)}$

where,

$\Delta(\Delta Ct) = \Delta Ct_{(sample)} - \Delta Ct_{(control)}$

Construction of deletion, complementation, and overexpression strains

A deletion mutant lacking *aslA* in the *A. nidulans* genome was constructed with targeted mutagenesis at its open reading frame (ORF). The chromosomal location of the ORF and its DNA sequence information was obtained from the public *A. nidulans* genome database (http://www.aspergillusgenome.org). The deletion cassette *aslA::argB* containing *argB* gene as a selection marker and the 5′- and 3′-flanking region of *aslA* ORF ($aslA_{orf}$) was constructed by double-joint PCR using the following primer pairs: PaslA-5f /PaslA-5r for the 5′-flanking region of $aslA_{orf}$; PaslA-3f /PaslA-3r for the 3′-flanking region of $aslA_{orf}$; PargB-f/PargB-r for the *argB* gene; and PaslA-5fn/PaslA-3rn for the final PCR round (Table 2). *A. nidulans* TJ1 was then transformed with the deletion cassettes and the resulting transformants were randomly screened for deletion of *aslA* by PCR and confirmed by Southern blotting (data not shown) to yield the Δ*aslA* strain (MCBA101; Table 1).

To complement the Δ*aslA* mutation, a 2.1-kb DNA fragment containing the 1.0-kb presumptive promoter *aslA(p)* and $aslA_{orf}$ was amplified from the genomic DNA of FGSC4 by PCR using the primers, PaslA-f and PaslA-r (Table 2). The resulting amplicon was cloned into the pT7Blue(R) (Novagen) to yield pT7-aslA. The 2.1-kb *Kpn*I-*Hin*dIII fragment containing the presumptive promoter region was excised from pT7-aslA and cloned into *Kpn*I-*Hin*dIII-digested pBS-pyroA vector (Lee *et al.*, 2004) to yield pBS-aslA. After verifying the sequence of the cloned fragment, the final plasmid was used to transform the recipient Δ*aslA* strain (MCBA101). Prototrophic $pyroA^{+}$ transformants were then screened for complementation of the Δ*aslA* mutation by PCR, followed by confirmation of the candidates by Southern blotting (data not shown) to yield the C'Δ*aslA* strain (MCBA201; Table 1).

For construction of the *aslA*-overexpressing strain, *the* $aslA_{orf}$ was amplified from the genomic DNA of FGSC4 using the primers, PaslA-0f and PaslA-0r (Table 2). The resulting 1.1-kb $aslA_{orf}$ fragment was cloned into pT7Blue(R) to yield pT7-aslA$_{ORF}$. The 1.1-kb *Hin*dIII fragment of $aslA_{orf}$ was excised and cloned into *Hin*dIII-digested pBS-pyroA vector downstream of *alcA(p)* to yield pBS-aslA, which was used to transform the recipient strain TJ1. The resulting $pyroA^{+}$ transformants were screened for integration of the *aslA* overexpression cassette into the genome by PCR, and the candidates were confirmed by Southern blotting (data not shown) to yield the overexpression strain OE *aslA* (MCBA301; Table 1). Reference strains that are isogenic to the mutants described above, except for the *aslA* locus, were constructed by crossing

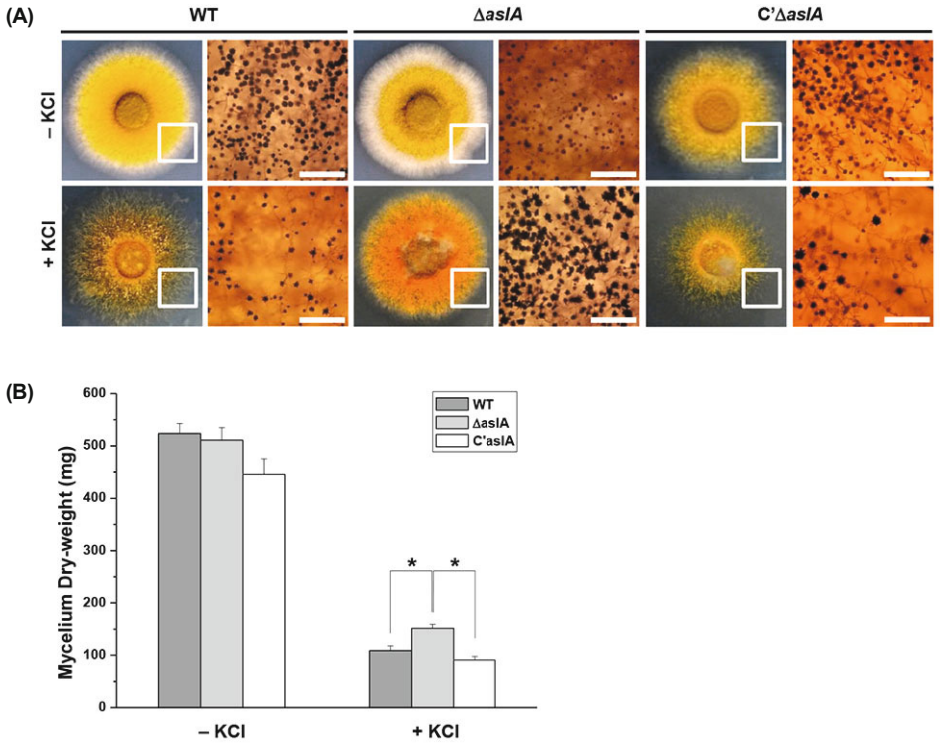

Fig. 2. Deletion of *aslA* attenuates the inhibitory effect of K^{+} stress on growth and asexual development. (A) *A. nidulans* strains were spot-inoculated and cultured on MMG (-KCl) and MMGK (+KCl) agar plates for 2 days, and the colony morphology was observed by stereomicroscopy. Bar, 100 μm. (B) *A. nidulans* strains were inoculated and cultured with shaking at 120 rpm for 2 days in 100 ml of liquid MMG (-KCl) and MMGK (+KCl) media, and mycelial dry-weights were measured. For each strain/culture condition, three independent biological replicates were performed. Values are mean ± SE from three independent experiments. * P <0.01 (Student's t test versus WT or C'Δ*aslA*).

the TJ1 strain with the FGSC4 strain, followed by screening for the segregant with one of the WT alleles of the auxotrophic markers, *argB*$^+$ (MCBA001; Table 1) or *pyroA*$^+$ (MCBA002; Table 1).

Microscopy

Slide-cultured mycelial samples were dually stained with 7-amino-4-chloromethylcoumarin (CMAC; Molecular Probes) and N-(3-thiethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl) pyridinium dibromide (FM4-64; Molecular Probes). The coumarin-based vacuole marker CMAC is selectively sequestered into vacuoles, which stains the vacuolar lumen, and the styryl dye FM4-64 specifically stains the vacuolar membrane under steady-state conditions with red fluorescence (Vida and Emr, 1995). Dual staining was performed as described previously (Shoji *et al.*, 2006) with some modifications. Briefly, the mycelia grown under the coverslips were stained with 5 μM FM4-64 at room temperature for 5 min, washed with 10 mM phosphate buffered saline (PBS, pH 7.4), and incubated in fresh MMG at 25°C for 50 min. Then the mycelia were submerged in MMG containing 10 μM CMAC and incubated at 30°C for 30 min, followed by washing with 10 mM PBS (pH 7.4). Finally, the mycelia were incubated in fresh MMG at 37°C for another 30 min.

For differential interference contrast (DIC) and fluorescence microscopy, an Olympus System microscope Model BX52 (Olympus) equipped with UPlanApo 40× and 100× objective lenses (Olympus) was used. U-MWIG3 (BP 530-550 excitation filter, DM 570 dichromatic mirror, LP 575 emission filter) and U-MWU2 (BP 330-385 excitation filter, DM 400 dichromatic mirror, BA 420 emission filter) Fluorescence Filter Cubes (Olympus) were used to observe the fluorescence of FM4-64 and CMAC, respectively. Images were processed and analyzed with Photoshop CS5.1 (Adobe Systems) and Image J (National Institutes of Health) software.

Results

aslA encodes a novel C_2H_2-type zinc finger transcription factor affecting K^+ stress tolerance

We have previously performed transcriptome profiling of *A. nidulans* and found that more than fifty transcription factor (TF) genes exhibited statistically significant variation in transcription throughout its asexual lifecycle (manuscript in preparation). Subsequently, a series of deletion mutants lacking the TF genes with peak expression occurring during asexual differentiation was constructed and primary phenotypic screening of the mutants was performed. Among the mutants, the deletion mutant of aslA (AN5583) showed enhanced K^+ stress tolerance compared to the WT strain (MCBA001; Table 1). Analysis of the *A. nidulans* genome database (http://www.aspergillusgenome.org) indicated that aslA is located on chromosome V. The deduced amino acid sequence of AslA consists of 306 amino acids (Mr 35.6 kDa) that contain tandem C_2H_2 zinc fingers (aa 27–54 and 57–83) near the N-terminus and a Gln-rich domain in the posterior portion (aa 245–281) (Fig. 1A). Protein database searches and phylogenetic analysis based on alignment of protein amino acid sequences revealed that proteins sharing significant sequence similarity with AslA are mainly present in the members of the family Trichocomaceae, including the genera *Aspergillus*, *Penicillium*, and *Talaromyces* (Fig. 1A and B).

Deletion of *aslA* attenuates the inhibitory effect of K^+ stress on growth and asexual development

The role of the *aslA* gene in K^+ stress tolerance was addressed by monitoring the growth and development of Δ*aslA* mutant on MMG and MMGK containing 1.2 M KCl in comparison with those of the WT and C'Δ*aslA* (MCBA201; Table 1) strains. After 2 days of culture on MMG plates, the Δ*aslA* mutant produced a reduced number of conidiophores compared to the WT and C'Δ*aslA* strains, although it showed a similar rate of radial growth as the latter (Fig. 2A). On MMGK

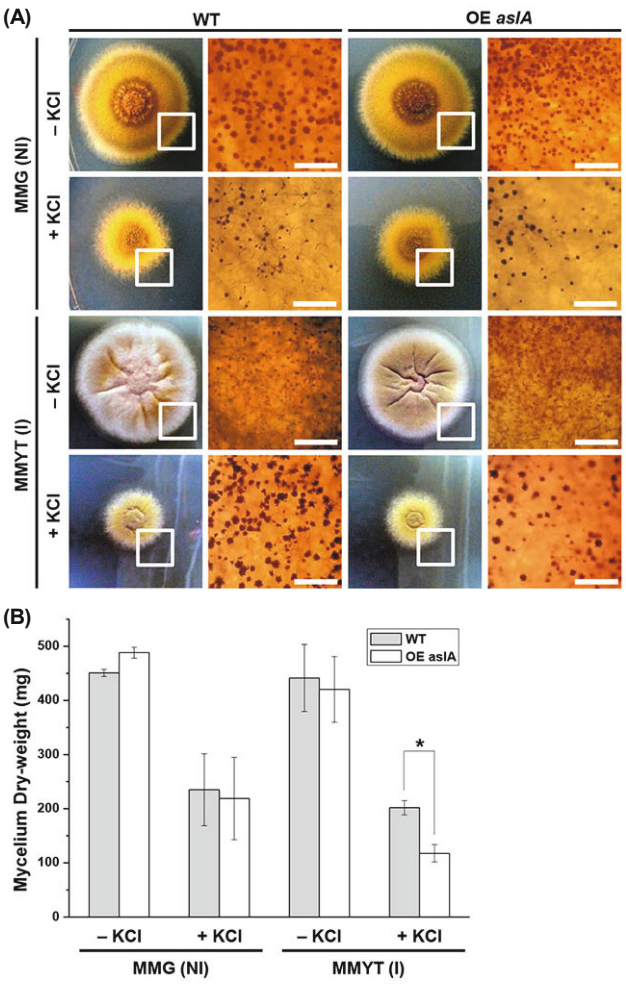

Fig. 3. Overexpression of *aslA* enhances the inhibitory effect of K^+ stress on growth and asexual development. (A) *A. nidulans* strains were spot-inoculated and cultured on MMG (-KCl), MMGK (+KCl), MMYT (-KCl), and MMYTK (+KCl) agar plates for 2 days, and the colony morphology was observed by stereomicroscopy. Bar, 100 μm. (B) *A. nidulans* strains were inoculated and cultured with shaking at 120 rpm for 2 days in 100 ml of liquid MMG (-KCl), MMGK (+KCl), MMYT (-KCl), and MMYTK (+KCl) media, and mycelial dry-weights were measured. I, inducing medium; NI, non-inducing medium. For each strain/culture condition, three independent biological replicates were performed. Values are mean ± SE from three independent experiments. * P <0.05 (Student's t-test versus WT).

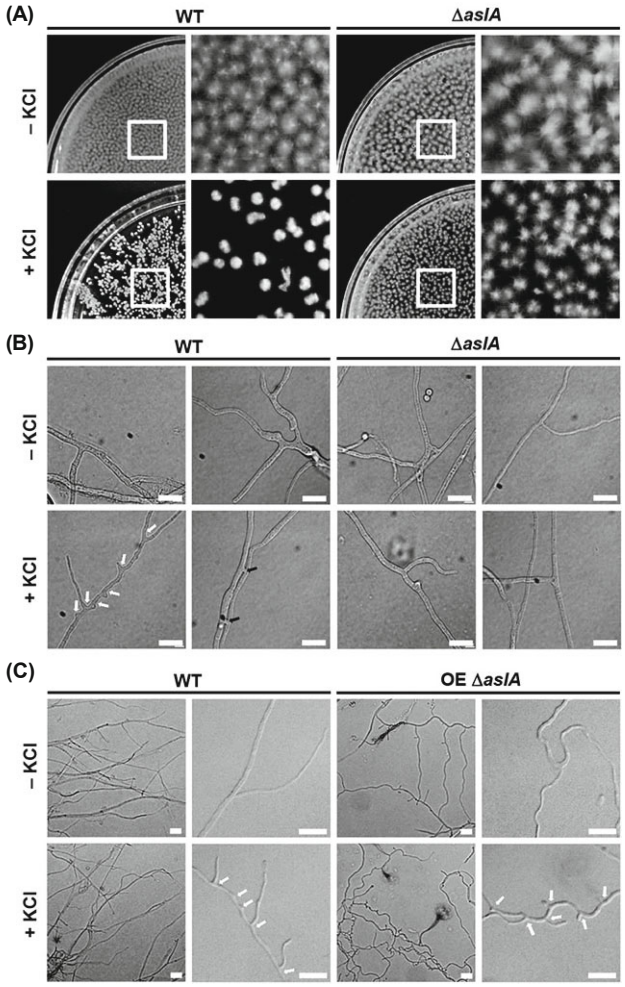

Fig. 4. Deletion of *aslA* alleviates K^+ stress-induced hyper-branching and curved growth. (A) *A. nidulans* strains were inoculated and cultured with shaking at 120 rpm in liquid MMG (-KCl) and MMGK (+KCl) media for 18 h, and the morphology of mycelial balls was observed. (B) *A. nidulans* strains were slide-cultured on the blocks of MMG (-KCl) and MMGK (+KCl) media for 2 days, and observed by DIC microscopy. (C) *A. nidulans* strains were slide-cultured on the blocks of MMYT (-KCl), and MMYTK (+KCl) media for 2 days, and observed by DIC microscopy. Bar, 20 μm. Arrows indicate branching points.

plates, however, the Δ*aslA* mutant developed a larger colony with a significantly higher density of conidiophores than the WT and C'Δ*aslA* strains. In agreement with this result, the dry cell weight of the Δ*aslA* mutant produced after 2 days of submerged culture in MMGK was about 40% higher than those of the WT and C'Δ*aslA* strains (Fig. 2B).

The effect of *aslA* overexpression in the WT background on growth and development under K^+ stress conditions was also observed using the OE *aslA* strain. On MMYTK plates, the OE *aslA* strain produced a reduced number of conidiophores compared to the WT strain (Fig. 3A). On the other hand, no apparent phenotypic differences were observed between the OE *aslA* and WT strains on MMYT, MMG, and MMGK plates. After 2 days of submerged culture, the OE *aslA* strain showed an approximately 40% reduction in vegetative growth in liquid MMYTK medium, but little difference in MMYT, MMG, and MMGK media when compared to the WT strain (Fig. 3B). These results together suggest that *aslA* contributes to the process of conidiophore development under normal conditions, but negatively affects the growth and asexual development at elevated concentrations of KCl.

Deletion of *aslA* alleviates K^+ stress-induced hyper-branching and curved growth

To understand the role of the *aslA* gene in hyphal branching and tip-extension, the effect of *aslA* deletion on the K^+ stress-induced morphological change of mycelial balls in submerged cultures was observed. While the WT strain produced significantly more compact mycelial balls in the submerged culture in the presence of 1.2 M KCl (MMGK) than in its absence (MMG), the Δ*aslA* mutant produced apparently normal mycelial balls regardless of the presence or absence of the extra salt (Fig. 4A). Consistent with this result, the WT strain produced hyper-branched hyphae and, in addition, showed hyphal fusion (anastomosis) on the KCl-supplemented solid medium (MMGK) (Fig. 4B). On the contrary, the Δ*aslA* mutant exhibited no significant change in hyphal branching caused by the presence of 1.2 M KCl (Fig. 4B).

The effect of *aslA* overexpression on the K^+ stress-induced morphological change was also observed using the OE *aslA* strain. When the overexpression of *aslA* was induced by threonine on solid media, the OE *aslA* strain produced curved hyphae both in the absence (MMYT) and presence (MMYTK) of 1.2 M KCl, though increased curvature of hyphae was observed on MMYTK (Fig. 4C). In addition, the K^+ stress-induced hyper-branching of hyphae was also observed in both the WT and OE *aslA* strains. These results together demonstrate that *aslA* plays a role in hyper-branching and curvature of hyphae under the K^+ stress conditions.

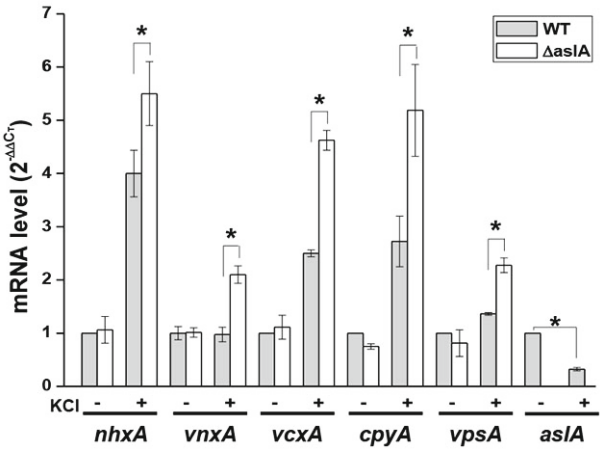

Fig. 5. Deletion of *aslA* enhances K^+ stress-inducible expression of the genes encoding proteins required for vacuolar biogenesis and sequestration of K^+. Total RNAs were extracted from the mycelia of *A. nidulans* strains that were harvested from the culture grown for 15 h in liquid CM and shifted to liquid MMG (-KCl) and MMGK (+KCl) for 30 min. qRT-PCR was performed using the primers for the genes of interest (*nhxA*, *vnxA*, *vcxA*, *cpyA*, *vpsA*, and *aslA*) and the internal control (*acnA*) listed in Table 2. Values are mean ± SE from three independent experiments. * P <0.05 (Student's t-test versus WT).

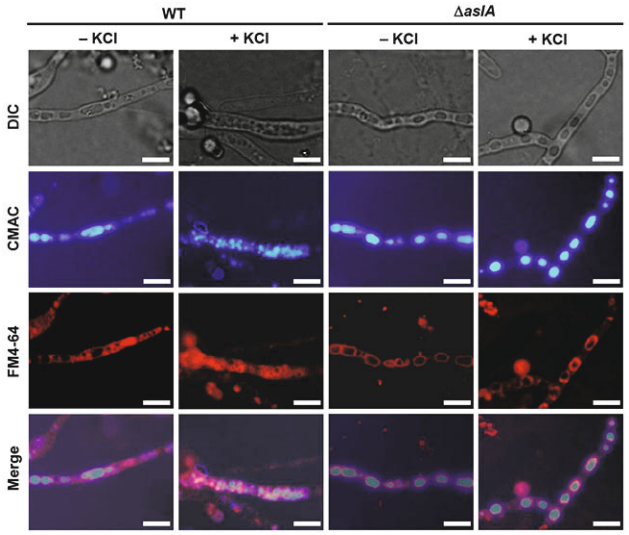

Fig. 6. Deletion of *aslA* prevents vacuolar fission and leads to swollen vacuoles in the cells exposed to K^+ stress. (A) *A. nidulans* strains were slide-cultured on the blocks of MMG (-KCl) and MMGK (+KCl) media for 2 days, and observed by DIC and fluorescence microscopy after specific labeling of the vacuolar lumen and membrane with CMAC and FM4-64, respectively. Bar, 5 μm.

Deletion of *aslA* enhances K^+ stress-inducible expression of the genes encoding the proteins required for vacuolar biogenesis and sequestration of K^+

The resistance of the Δ*aslA* mutant to K^+ stress implicated that the novel transcription factor AslA may be involved in ion homeostasis by regulating the expression of the genes encoding vacuolar/prevacuolar transporters for alkali metal ions. To investigate the role of AslA in transcriptional regulation of the vacuole-related genes, we first monitored the change of *aslA* expression in response to K^+ stress in the WT strain by qRT-PCR. When shifted from CM to MMGK, which contained 1.2 M KCl (30 min incubation), the level of *aslA* expression was decreased to one third of the original level (Fig. 5). We also estimated the level of K^+ stress-inducible expression of the putative vacuolar/prevacuolar transporter genes, *nhxA* (AN2288), *vnxA* (AN6986), and *vcxA* (AN0471), as well as those encoding the proteins involved in vacuolar biogenesis, *cpyA* and *vpsA*, in the WT and Δ*aslA* strains. When shifted from liquid CM to MMG, the mycelia of Δ*aslA* and WT strains showed similar expression levels of all five genes (Fig. 5). On the other hand, the Δ*aslA* mutant showed a significantly higher level of K^+ stress-inducible transcription of the five genes compared to the WT strain when its mycelia were shifted to MMGK. These results suggest that the transcription factor AslA functions as a negative regulator of the K^+ stress-inducible expression of the vacuolar/prevacuolar transporter genes, as well as those encoding the proteins required for vacuolar biogenesis.

Deletion of *aslA* prevents vacuolar fission and leads to swollen vacuoles in the cells exposed to K^+ stress

The data suggesting the negative regulatory role of *aslA* in K^+ stress-inducible expression of the vacuole-related genes (Fig. 5) raised a possibility that *aslA* can contribute to the changes in vacuolar morphology caused by exposure of the fungal cells to K^+ stress. Thus, we observed a morphological change of vacuoles in the slide-cultured mycelia of the Δ*aslA* and WT strains exposed to K^+ stress by microscopy after specific labeling of the vacuolar lumen and membrane with CMAC and FM4-64, respectively. On MMG medium, both the Δ*aslA* and WT strains showed a mainly fused form of vacuoles, and thus little difference was found in their vacuolar morphology (Fig. 6). However, on MMGK containing 1.2 M KCl, the WT strain showed multiple smaller vacuoles, whereas the Δ*aslA* mutant showed no signs of vacuolar fission and exhibited unfragmented vacuoles as on the medium without supplemented KCl. These results indicate that on exposure of mycelia to high concentrations of KCl, *aslA* deletion prevents vacuolar fission and leads to persistence of unfragmented vacuoles. Furthermore, it seems likely that the enhanced K^+ stress-inducible expression of the vacuole-related genes caused by *aslA* deletion is responsible for the changes in vacuolar morphology.

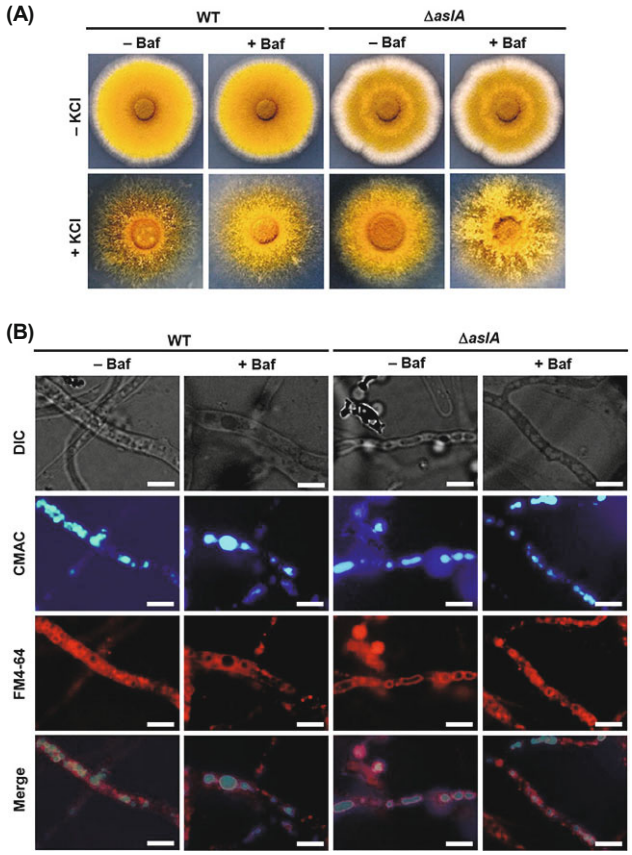

Fig. 7. The increased K^+ stress resistance and the prevention of K^+ stress-inducible vacuolar fission caused by *aslA* deletion are dependent on the activity of V-ATPase. (A) *A. nidulans* strains were spot-inoculated and cultured on MMG (-KCl) and MMGK (+KCl) agar plates with (+ Baf) or without bafilomycin (-Baf) for 2 days, and the colony morphology was observed. (B) *A. nidulans* strains were slide-cultured on the blocks of MMGK (+KCl) media with (+Baf) or without bafilomycin (-Baf) for 2 days, and observed by DIC and fluorescence microscopy after specific labeling of the vacuolar lumen and membrane with CMAC and FM4-64, respectively. Bar, 5 μm.

The increased K^+ stress resistance and the prevention of K^+ stress-inducible vacuolar fission caused by *aslA* deletion are dependent on the *activity* of V-ATPase

In *S. cerevisiae*, all three vacuolar/prevacuolar cation/H^+ antiporters, Nhx1, Vnx1, and Vcx1, are involved in extrusion of excess K^+ and other metal ions from the cytosol into vacuoles via a secondary transport process which is energy-dependent and driven by the proton-motive force generated by V-ATPase (Cagnac *et al.*, 2007, 2010; Qiu, 2012). Thus, we were curious to know whether the increased K^+ stress resistance of the Δ*aslA* mutant mediated by the elevated expression of the putative cation/H^+ antiporters, NhxA, VnxA, and VcxA, is dependent on the proton pumping activity of V-ATPase.

The Δ*aslA* and WT strains showed significantly different responses to the V-ATPase inhibitor, bafilomycin, under K^+ stress conditions. On MMGK supplemented with 25 μM bafilomycin, the increased resistance of Δ*aslA* mutant to K^+ stress was considerably attenuated (Fig. 7A). On the other hand, the WT strain exhibited slightly better growth and development in the presence of bafilomycin than in its absence. When the morphological change of vacuoles was observed by DIC and fluorescence microscopy, the Δ*aslA* mutant showed a larger number of fragmented vacuoles in the presence of bafilomycin than in its absence, while the WT mycelia showed unfragmented vacuoles in addition to fragmented ones on exposure to bafilomycin (Fig. 7B). These results together suggest that the increased K^+ stress resistance and maintenance of unfragmented vacuoles seen in the Δ*aslA* mutant are mediated at least partially by the elevated expression of the putative V-ATPase-dependent cation/H^+ antiporters, NhxA, VnxA, and VcxA. On the contrary, the inhibition of V-ATPase by bafilomycin caused enhanced vacuolar fusion and increased K^+ resistance to some extent in the WT strain. This result is in agreement with a previous report demonstrating that elimination of proton pump activity by the V-ATPase either pharmacologically or by conditional or constitutive V-ATPase mutations blocks salt-induced vacuole fragmentation in *S. cerevisiae* (Baars *et al.*, 2007).

Discussion

We found in the present study that the *aslA* gene encoding a novel C_2H_2-type zinc finger TF negatively contributes to the resistance to K^+ stress and to the persistence of unfragmented vacuoles under K^+ stress conditions. We also revealed that AslA attenuates the K^+ stress-inducible expression of the genes that are supposed to encode the endosomal and vacuolar cation/H^+ exchangers powered by V-ATPase, as well as those encoding the proteins involved in vacuolar biogenesis and function.

The initial clue as to the involvement of *aslA* in K^+ stress tolerance was based on the results that the Δ*aslA* mutant showed better growth and development in the presence of 1.2 M KCl than the WT strain (Fig. 2). In addition, the OE *aslA* strain showed reduced growth and conidiophore development in the presence of 1.2 M KCl than the WT strain, when *alcA* promoter-controlled *aslA* expression was induced by threonine (Fig. 3). We furthermore found that deletion of *aslA* alleviates the K^+ stress-induced hyper-branching and curved growth of hyphae observed in the WT and OE *aslA* strains (Fig. 4). These results together led us to conclude that *aslA* plays a negative role in establishing K^+ stress tolerance in the presence of high concentrations of KCl. Based on this finding, we attempted to address whether the function of *aslA* is related to the resistance against other alkali metal ion-induced stress or hyperosmotic stress. The Δ*aslA* mutant exhibited higher rates of growth and development than the WT strain in the presence of 1.2 M NaCl, but not in the presence of 2.4 M sorbitol (data not shown). Thus, we could assume that the function of *aslA* is involved in the resistance to the alkali metal ions, K^+ and Na^+, but not to osmostress.

It is essential for living cells to maintain appropriate intra-

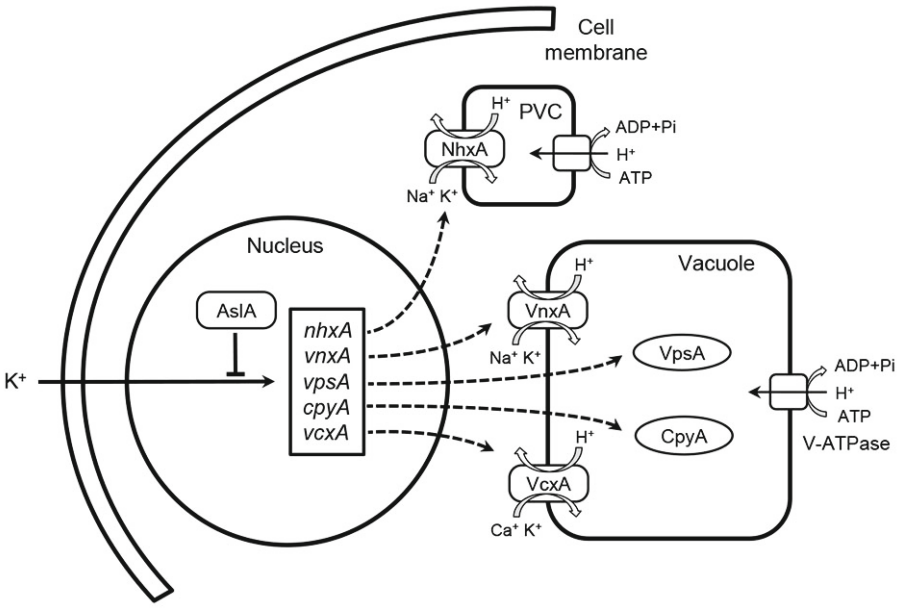

Fig. 8. A simplified view of the negative regulation of the vacuole-mediated resistance to K^+ stress by AslA in *A. nidulans*. AslA negatively affects the K^+ stress-inducible expression of the genes of the vacuolar/prevacuolar cation/H^+ exchangers as well as those encoding the proteins required for vacuolar biogenesis. All the three vacuolar/prevacuolar cation/H^+ exchangers, NhxA, VnxA, and VcxA, are expected to have the potential for V-ATPase-dependent sequestration of excess cytosolic K^+ ion into vacuoles. VpsA is a putative dynamin-related protein that is essential for vacuolar biogenesis, and CpyA is the representative vacuolar cargo protein, carboxypeptidase Y.

cellular concentrations of alkali metal cations, principally K^+ and Na^+, since they determine cell volume, intracellular pH, and electrical potential across the plasma membrane. One of the main strategies employed by yeast cells to maintain an optimum intracellular concentration of K^+ and a stable and high intracellular K^+/Na^+ ratio is selective compartmentalization of toxic or surplus cations in vacuoles (Li and Kane, 2009; Arino *et al.*, 2010). In this context, we speculated that the resistance of Δ*aslA* mutant to K^+ stress might be due to altered expression of the genes involved in vacuolar function or biogenesis. In agreement with our expectation, the qRT-PCR results suggest that AslA attenuates the K^+ stress-inducible expression of *nhxA*, *vnxA*, and *vcxA* genes that are supposed to be the homologues of the yeast genes, *NHX1*, *VNX1*, and *VCX1*, respectively, which encode the endosomal and vacuolar cation/H^+ exchangers powered by V-ATPase (Fig. 5). This result is in good agreement with the enhanced K^+ stress tolerance of the Δ*aslA* mutant in that all the putative cation/H^+ exchangers (NhxA, VnxA, and VcxA) encoded by the three genes subjected to AslA-mediated negative transcriptional regulation have the potential for sequestration of K^+ within the vacuolar lumen. Considering that all three of the putative transporters are thought to have the potential for sequestration of excess cytosolic K^+ ion into vacuoles, it seems reasonable that deletion of *aslA* enhances the K^+ stress resistance of fungal cells by increasing the K^+ ion exchange capacity of the vacuolar/prevacuolar membrane. In addition, the enhanced K^+ stress resistance of the Δ*aslA* mutant can also be due to the increased K^+ stress-inducible production of the vacuolar proteins, VpsA (Tarutani *et al.*, 2001) and CpyA (Ohsumi *et al.*, 2001), causing increases in the storage capacity or activity of vacuoles. On the other hand, we could not identify any meaningful change in the levels of inducible expression of osmostress-response genes, such as *hogA* (Hagiwara *et al.*, 2014), *srrA* (Hagiwara *et al.*, 2007), *atfA* (Balazs *et al.*, 2010), and *gfdA* (Fillinger *et al.*, 2001) (data not shown), indicating that *aslA* is irrelevant to the process for acquisition of tolerance against hyperosmotic stress.

We found involvement of *aslA* in the morphological change of vacuoles in response to elevated concentrations of K^+. On exposure to K^+ stress, the Δ*aslA* mutant showed unfragmented vacuoles, while the WT strain showed highly fragmented ones (Fig. 6), and the mutant phenotype was suppressed by the inhibitor of V-ATPase, bafilomycin (Fig. 7). Thus, the Δ*aslA* mutant probably owed its improved K^+ stress resistance to the decreased tendency of vacuolar fission and increased K^+ sequestration capacity, which resulted at least partially from the enhanced K^+ stress-induced expression of the V-ATPase-dependent vacuolar/prevacuolar cation/H^+ antiporters, NhxA, VnxA, and VcxA.

V-ATPase performs two distinct roles in vacuole fission and fusion in *S. cerevisiae*: fusion requires the physical presence of the membrane sector of V-ATPase, while fission, in contrast, depends on its proton translocation activity (Baars *et al.*, 2007). Thus, the dual involvement of V-ATPase in vacuole fission and fusion suggests its role as a potential regulator of vacuolar morphology and membrane dynamics. Consistent with the previous findings in *S. cerevisiae*, our data suggest that inhibition of the proton pumping activity of V-ATPase by bafilomycin results in increased vacuole fusion and K^+ resistance in the WT strain of *A. nidulans* (Fig. 7). However, in the Δ*aslA* mutant, inhibition of V-ATPase by bafilomycin caused considerable attenuation of the mutant phenotype: increased resistance and maintenance of unfragmented vacuoles against K^+ stress. Therefore, it is suggested that there exists some distinct mechanism of the contribution of V-ATPase to vacuolar morphogenesis between the WT and Δ*aslA* strains of *A. nidulans* that remains to be addressed.

The phenotype of the Δ*aslA* mutant of increased K^+ stress resistance and persistence of unfragmented vacuoles under K^+ stress conditions is not likely to be fully suppressed by the inhibitor of V-ATPase, bafilomycin (Fig. 7). It is thus suggested that the increased K^+ stress-inducible expression of *vpsA* and *cpyA* (Fig. 5) also contributes to the phenotype of Δ*aslA* mutant under K^+ stress conditions (Fig. 6). This view seems to be reasonable, considering that the yeast homologue of VpsA, Vps1, is a dynamin-related protein essential for vacuolar biogenesis (Tarutani *et al.*, 2001), and that CpyA is a representative vacuolar cargo protein, carboxypeptidase Y (Ohsumi *et al.*, 2001).

Consequently, we can present a simplified view of the proposed role of AslA in the K^+ stress-inducible expression of the genes of the vacuolar/prevacuolar transporters, as well as those encoding the proteins required for vacuolar biogenesis, as shown in Fig. 8. To the best of our knowledge, this is the first report indicating that the function of a C_2H_2-type zinc finger TF is associated with K^+ stress resistance and vacuolar morphogenesis via the transcriptional regulation of the vacuole-related genes in filamentous fungi. However, many questions remain unsolved regarding the molecular genetic mechanism of the AslA-dependent regulation of the vacuole-mediated resistance to K^+ stress in *A. nidulans*.

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