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ORIGINAL PAPER

The *anisin1* gene encodes a defensin-like protein and supports the fitness of *Aspergillus nidulans*

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Abstract In the genome of *Aspergillus nidulans*, a defensin-like protein, Anisin1, was annotated with high homology to the mosquito defensin AaDefA1. So far, no studies exist on defensins from filamentous ascomycetes. Therefore, we characterized the *anisin1* gene in *A. nidulans* and generated a deletion mutant, which suffered from a defect in mitospore development and produced less conidia at 42°C compared to the reference strain. In surface cultures of *A. nidulans* wild type, the *anisin1* expression correlated with that of the central regulator for asexual development, *brlA*, and with the major scavanger of H_2O_2 stress, *catB*, which is indicative for cell differentiation in developing fungi. Interestingly, *brlA* and *anisin1* expressions were deregulated in a Δ*srrA* strain that covers a central role in the histidine-to-aspartate (His-Asp) phosphorelay signaling pathway and shows impaired asexual development and H_2O_2 detoxification. In submers cultures of *A. nidulans* wild type and other mutants of the His-Asp phosphorelay signaling pathway, *anisin1* was repressed, but derepressed in a Δ*srrA* background, and *anisin1* transcription was further increased in this mutant by H_2O_2 addition. We therefore conclude that the secreted protein Anisin1 contributes to the optimal development of *A. nidulans* and we further propose that it has a sensing/signaling function for elevated H_2O_2 levels.

Keywords Defensin · Asexual development · Oxidative stress · *Aspergillus nidulans*

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Introduction

Antimicrobial peptides (AMPs), such as defensins, are widely distributed in nature as they can be found in organisms ranging from bacteria, plants, insects to humans where they are the product of gene transcription and translation of a single gene (Raj and Dentino 2002; Aerts et al. 2008). In higher eukaryotes, the synthesis of AMPs occurs constitutively or after infection or injury and forms a first line of defense against invading pathogens. Importantly, AMPs represent inducible effector molecules that act as modulators of the innate immune system beside their antimicrobial toxicity (Gallo et al. 2002; Hancock and Patrzykat 2002; Raj and Dentino 2002; Toke 2005; Aerts et al. 2008).

The role of AMPs of prokaryotes and lower eukaryotes is less well understood. Lacking an immune system comparable to that of higher eukaryotes, AMPs could contribute to an ecological advantage of these microorganisms when exposed to unfavorable environmental conditions (Marx 2004). Indeed, bacterial AMPs are part of the quorum sensing mechanism, which plays an important role in microorganisms to communicate and coordinate their behavior by accumulating diffusible signaling molecules in the extracellular environment. This enables, for example, bacteria to chemically measure their cell density and to

regulate numerous cellular processes such as secretion of virulence factors, production of biofilms, transcriptional activation of AMPs and oxidative stress resistance (Raina et al. 2009). In analogy, AMPs of filamentous ascomycetes, such as PAF from *Penicillium chrysogenum* or AFP from *Aspergillus giganteus*, have antifungal activities (Kaiserer et al. 2003; Theis et al. 2003) and are developmentally regulated (Meyer et al. 2002; Hegedüs et al. 2011b). We could recently show that PAF exhibits an important function in the modulation of asexual development (Hegedüs et al. 2011b).

Defensins represent a unique family of small, cysteine-rich and structured peptides (Raj and Dentino 2002). They consist of about 28–44 amino acids (aa) and have a low molecular mass (3–5 kDa). Up to eight cysteine residues participate in intramolecular disulfide bonds that stabilize an antiparallel β-sheet and an α-helix, (Dimarcq et al. 1998; Gallo et al. 2002; Thevissen et al. 2007; Verma et al. 2007; Aerts et al. 2008). In silico analysis indicated that putative defensin orthologous genes are present also in the genomes of filamentous ascomycetes (Zhu 2008), but they have not been characterized in this fungal group so far.

In this study, we started to investigate the function of a defensin-like encoding gene from the model organism *Aspergillus nidulans*, *anisin1* (AN5046.3; Zhu 2008). The gene product Anisin1 shows high homology to AaDefA1, a defensin from the blood feeding mosquito *Aedes aegypti*.

By the characterization of an *anisin1* deletion mutant in *A. nidulans*, we provide for the first time evidence that *anisin1* plays an important role in the asexual development of *A. nidulans* by supporting conidiation, especially at elevated cultivation temperatures. Expression analyses in wild-type cells and mutants of the histidine-to-aspartate (His-Asp) phosphorelay signaling cascade indicate that *anisin1* transcription is derepressed in a mutant (Δ*srrA*) that suffers from impaired asexual development, which further underlines a developmental function of *anisin1*. Moreover, the expression pattern of *anisin1* in Δ*srrA*, which also suffers from defective H_2O_2 detoxification, provides first data that Anisin1 may be involved in sensing/signaling elevated H_2O_2 levels.

Materials and methods

Strains, media and culture methods

The *A. nidulans* strains used in this work are listed in Table S1 (Supplements). Generally, fungal strains were grown at 37°C on solid complete medium (CM; 2% D-glucose, 0.2% peptone, 0.1% yeast extract, 0.1% NZ-amine A, 0.05% KCl, 0.04% $MgSO_4 \cdot 7H_2O$, 0.15% KH_2PO_4, pH 6.5) with the appropriate supplements. Alternatively, minimal medium (MM) according to Pontecorvo et al. (1953) with appropriate supplements was used. To synchronize surface cultures, 18-h-old submers precultures (200 ml MM) were harvested by filtration, transferred to solid MM and further incubated at 37°C. To test oxidative stress response, 18-h-old submers cultures were exposed to 20 mM H_2O_2 for 2 h before harvesting. For crossfeeding experiments, 5×10^5 conidia (in 50 µl) of *A. nidulans* Δ*ku70* and Δ*anisin1* were spotted on 30-ml solid MM and incubated for 38–48 h at 42°C.

Deletion of *anisin1* and complementation

To delete the *anisin1* gene in *A. nidulans*, the plasmid pSK275 was used, containing the pyrithiamine (*ptrA*) expression cassette (Kubodera et al. 2002). According to the splitmarker method (Nielsen et al. 2006), *A. nidulans* TN02A3 (Δ*ku70*) was co-transformed with two DNA constructs, each containing an incomplete fragment of the *ptrA* expression cassette fused to 1.168 and 1.199 bp of the *anisin1* gene flanking sequences, respectively. Homologous integration of each fragment into the flanking sites of *anisin1* allowed recombination of the *ptrA* fragments, which shared a 560 nucleotide overlap, and resulted in the generation of the intact *ptrA* resistance gene at the site of recombination.

The two DNA constructs for the co-transformation were prepared as follows: the 5′-UTR fragment and 3′-UTR fragment of *anisin1* were PCR amplified from genomic DNA (gDNA) with the specific primers 5′ UTR forward B and 5′ UTR reverse and 3′ UTR forward and 3′ UTR reverse (Supplements, Table S2). The PCR fragments of 1.629 bp (5′-UTR fragment) and 1.596 bp (3′-UTR fragment) in size were digested with SacI (5′-UTR fragment) and HindIII (3′-UTR fragment), respectively. The *ptrA* selection marker was released from the plasmid pSK275 by digestion with SacI and HindIII and ligated to the 5′-UTR fragment and to the 3′-UTR. The two overlapping fragments were amplified from the ligation product using the primers 5′ UTR forward nested and *ptrA* reverse generating splitmarker I and *ptrA* forward and 3′ UTR reverse nested generating splitmarker II (Supplements, Table S2).

For complementation, a 4.114-bp *anisin1* gene construct was amplified from gDNA using the specific primers 5′ UTR forward A and 3′ UTR reverse and then ligated into pGEM-T (Promega). The *anisin1* fragment was released by NdeI digestion and ligated into the NdeI linearized pAN8.1 vector that carries the phleomycin (*ble*) resistance marker (Punt and van den Hondel 1992). The plasmid pAN8.1/*anisin1* was linearized by BsiWI and used for complementation of the deletion strain Δ*anisin1*.

Transformation of *A. nidulans* was carried out as previously described by Tilburn et al. (1983). *anisin1*-depleted transformants were prescreened three times for their ability

to grow on MM containing 100 µg/l pyrithiamine hydrobromide. Transformants complemented with the *anisin1* wild-type gene were prescreened three times for their ability to grow on MM containing 40 mg/l phleomycin.

Verification of gene deletion and complementation by Southern blot analysis

Site-specific, single-copy integration of transforming DNA was verified by Southern blot analysis according to the protocol of Sambrock et al. (1989). Digested gDNA was separated on a 0.7% agarose gel and blotted onto Hybond N membranes (Amersham, Biosciences). For verification of *anisin1* deletion, gDNA was restricted with NdeI and hybridized with the digoxigenin-dUTP-labeled *ptrA* and *anisin1* probes amplified by PCR with the respective oligonucleotides (Supplements, Table S2). For verification of the complementation, gDNA was digested with EcoRI and hybridized with a digoxigenin-dUTP-labeled probe spanning the 5′-UTR and half of the *anisin1* gene. Probes were detected with anti-digoxigenin-dUTP antibodies conjugated to alkaline phosphatase according to the manufacturer's instructions (Roche).

Viability of conidia and vegetative growth tests

Conidia were harvested from 48-h-old surface cultures and resuspended in spore suspension buffer (0.9% NaCl, 0.01% Tween 80). The spore suspension was then diluted to 1×10^5 conidia/ml and stored at 4°C with or without 15% glycerol for up to 12 days. Every 3 days (day 0, 3, 6, 9, 12), aliquots were diluted appropriately, spread on MM agar plates and further incubated at 37°C for 48 h. The number of colonies was determined and those at day 0 were used as a reference to calculate the survival rates (%).

Growth tests were performed in 6-well plates containing 5 ml appropriately supplemented solid MM (nitrogen sources: 20 mM ammoniumtartrate or $NaNO_2$, $NaNO_3$; carbon sources: 10% glucose, 50 or 200 mM sodium acetate). 2×10^3 conidia were point inoculated and incubated at 37°C. Growth was monitored by determining the colony diameters in a time course over 72 h. Growth was also monitored by determining the dry weight of the fungal strains when grown in liquid medium. To this end, 100 ml of CM and MM was inoculated with 1×10^8 conidia and incubated for 24 h at 37°C before the mycelia were harvested. All experiments were performed in duplicates and repeated twice.

Application of oxidative stress in agar plates

To determine the sensitivity of the *A. nidulans* Δ*anisin1* deletion mutant to oxidative stress–inducing compounds, agar diffusion assays were performed as described by Eisendle et al. (2006).

Briefly, 1×10^4 conidia were spread on solid MM. Then, holes of 0.5 cm in size were punched in every agar plate, and 50 µl of 20–100 mM H_2O_2, 1–10 mM menadione and 100–500 mM paraquat were applied. The plates were incubated at 37°C for 48 h, and the diameter of the growth inhibition zone was determined. All experiments were performed in duplicates and repeated twice.

Determination of conidial counts

To investigate mitospore production of the *anisin1* deletion strain, 2×10^3 conidia were point inoculated in 6-well plates containing 5-ml solid MM and incubated at 30, 37 and 42°C. Growth was monitored by determining the colony diameters in a time course over 72 h. Conidia were harvested by vortexing the excised surface area in spore suspension buffer and counted. The amount of conidiospores/cm^2 was calculated according to the formula: conidia/cm^2 = conidia per ml × volume/A whereby $A = r^2 \cdot \pi$. Mycelium from equivalent cultures was harvested and subjected to RNA isolation for Northern blot analysis. All experiments were set up in duplicates and were repeated twice.

Northern blot analysis

Total RNA was isolated using TRI reagent (Sigma-Aldrich). 5–10 µg of total RNA was separated on 1.2% formaldehyde agarose gels and blotted onto Hybond N membranes (Amersham, Biosciences) according to the protocols of Fourney et al. (1988) and Kroczek and Siebert (1990). The hybridization probes were generated and labeled with digoxigenin-dUTP (Roche) by PCR using gene-specific primers (Supplements, Table S2). Hybridization probes were detected with anti-digoxigenin-dUTP antibodies conjugated to alkaline phosphatase according to the manufacturer's instructions (Roche). The quantification of signal intensities of mRNAs on Northern blots was calculated and compared using the Image Quant 5.2 Software (Molecular Dynamics). 18S and 26S rRNA were used as internal standards. All experiments were repeated twice.

Results and discussion

In silico analysis

A tblastn search in the genome of *A. nidulans* identified two candidate genes with high homology to the insect defensin gene AaDefA1 of the mosquito *A. aegypti*. One was annotated by Sanger, Broad Institute, as a conserved

hypothetical protein on chromosome 3, which contains an arthropod defensin protein domain (contig 84; AN5046.3). This protein was named Anisin1 by Zhu (2008). The other was assigned as Atesin3 (Sanger, Broad Institute) and is localized on chromosome 1 (contig 108; AN11510.3). This protein corresponds to Anisin2 in Zhu (2008).

A ClustalW multiple alignment of the aa sequence of the premature protein forms of Anisin1 and AaDefA1 revealed that both exhibit 35% identity (Supplements, Figure S1). A signal peptide, a propeptide and the N-terminus of the putative mature protein of Anisin1 were identified (Kyte and Doolittle 1982; iPSORT, http://ipsort.hgc.jp/), indicating that Anisin1 is an extracellular protein. The mature proteins Anisin1 and AaDefA1 contain 41 and 40 aa, respectively, and exhibit 51% aa identity (Fig. 1). The postulated positions of the disulfide bonds between the six cysteine residues of the AaDefA1 also fit to Anisin1, which underlines the structural similarity between both the *A. nidulans* and the mosquito defensin (Zhu 2008) (Fig. 1). The aa sequence of Atesin3 and AaDefA1 showed an identity of 25% in their premature form and 46% identity after maturation (data not shown). Anisin1 and Anisin2/Atesin3 exhibit 69% identity as premature and 70% identity as mature proteins (data not shown).

In this study, we concentrated on the characterization of *anisin1* that showed the highest aa identity to the mosquito defensin AaDefA1. Analysis of the promoter region of *anisin1* revealed several putative regulatory elements that could be involved in the transcriptional regulation, for example, by the HAP-like complex (Brakhage et al. 1999), CREA (Kulmburg et al. 1993) and GATA factors (Scazzocchio 2000). Besides, four putative stress–responsive elements (STREs) are present (5′-CCCCT-3′ or 5′-AGGGG-3′; position −113, −816, −988 and −998), which might be involved in the activation of *anisin1* expression in response to signals related to oxidative stress, nutrient starvation, osmotic stress or low pH (Toone and Jones 1998). A TATAA box is missing (Supplements, Figure S2).

Anisin1 balances asexual development

To investigate the role of *anisin1* in *A. nidulans*, an *anisin1* knockout strain was constructed in a Δ*ku70* background (Supplements, Figure S3A–C), and growth and spore viability tests were performed to characterize the phenotype of the Δ*anisin1* mutant. No difference between the mutant strain and the reference strain Δ*ku70* could be detected in respect to the viability of conidia and the growth rate in liquid or on solid medium. Neither secondary carbon nor secondary nitrogen sources affected the growth of the deletion mutant compared to the control strain under the conditions tested (see "Materials and methods"; data not shown).

Next, we characterized the mitospore production in the *anisin1* deletion strain compared to Δ*ku70* on solid MM at 30, 37 and 42°C, respectively. After 48 h of incubation, there was little phenotypical difference between the mutant and the reference strain when grown at 30 and 37°C, with the exception that at 37°C the Δ*ku70* colony was covered with aerial hyphae which may represent the second generation (Fig. 2a; Supplements Table S3). A severe defect in mitospore development, however, was evident in the Δ*anisin1* strain at 42°C (Fig. 2a). To assess this phenomenon, the conidial counts were determined (Table 1). At 30°C, the number of conidia of the Δ*anisin1* strain was reduced by 10% after 48 h and 1% after 72 h and further decreased at 37°C by 15% after 48 h and 21% after 72 h. However, at 42°C the defect in conidia formation of the mutant culminated after 48 h in a reduction by 93% compared to Δ*ku70*. This defect in asexual development slightly ameliorated to −65% after 72 h of incubation compared to the reference strain. The complementation of *anisin1* rescued the defect in mitospore production (Table 1; Fig. 2a, Supplements Figure S4A, B) and confirmed that the identified phenotype resulted from the deletion of the *anisin1* gene. Furthermore, crossfeeding of Δ*anisin1* by co-cultivation with Δ*ku70* restored the defect

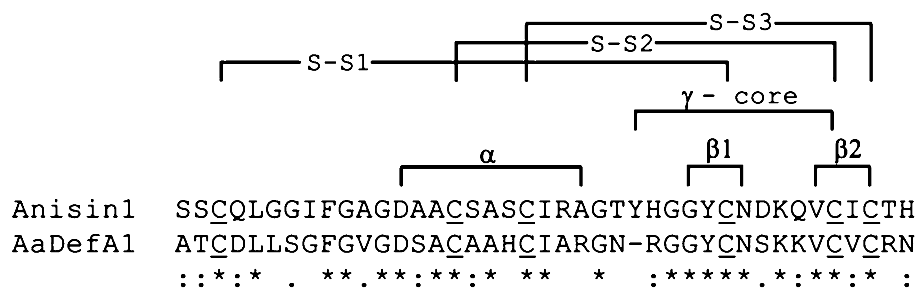

Fig. 1 Alignment of the aa sequences of the mature *A. nidulans* Anisin1 and *A. aegypti* insect defensin AaDefA1. Putative structural motifs (γ-core, the α-helix and the β-strands) and the proposed disulfide bond pattern of Anisin1 and AaDefA1 are indicated at the *top*. The cysteins involved in the formation of disulfide bridges (S–S) are *underlined*. Identical residues are marked with an *asterisk*, conserved substitutions with a *colon* and semi-conserved substitutions with a *dot*

in mitospore development at the point of contact of both colonies (Fig. 2b, left panel). This effect was less pronounced when Δ*anisin1* was co-cultivated with Δ*anisin1* (Fig. 2b, right panel). This suggests that Anisin1 is secreted by Δ*ku70* into the agar and rescues at least in part the sporulation defect of the Δ*anisin1* strain. However, other so far not defined factors support a slight sporulation independently from Anisin1 under the conditions tested.

The role of *anisin1* in asexual development was further corroborated by Northern blot analysis where we monitored the expression of *anisin1* and the central regulator for asexual development in *A. nidulans*, *brlA* (Adams et al. 1998), in point inoculated surface cultures of the mutant and the reference strain. In a time course over 48 h, *anisin1* and *brlA* were similarly expressed in a Δ*ku70* surface culture grown at 37 and 42°C (Fig. 3). The *anisin1* and *brlA* transcription was strongest after 24 h and decreased by 48 h. At 30°C, however, the transcription of *anisin1* and *brlA* was delayed, reflecting the lower proliferation and conidiation rate of *A. nidulans* at this temperature. The *anisin1* and *brlA* mRNAs were most abundant after 36 h of incubation and the transcription level of *anisin1* declined rapidly by 48 h of incubation whereas the level of *brlA* transcripts decreased only moderately at this time point. In the Δ*anisin1* mutant, the *brlA* expression at 30°C paralleled the expression in the reference strain (Fig. 3). The quantification of the Northern blot signal intensities of the respective mRNAs revealed, however, that at 37°C the *brlA* expression was moderately decreased in the mutant by 40% after 24 h, 63% after 36 h and 60% after 48 h compared to the reference strain (Fig. 3; Table 2). Note that the signal intensities of all samples were first corrected

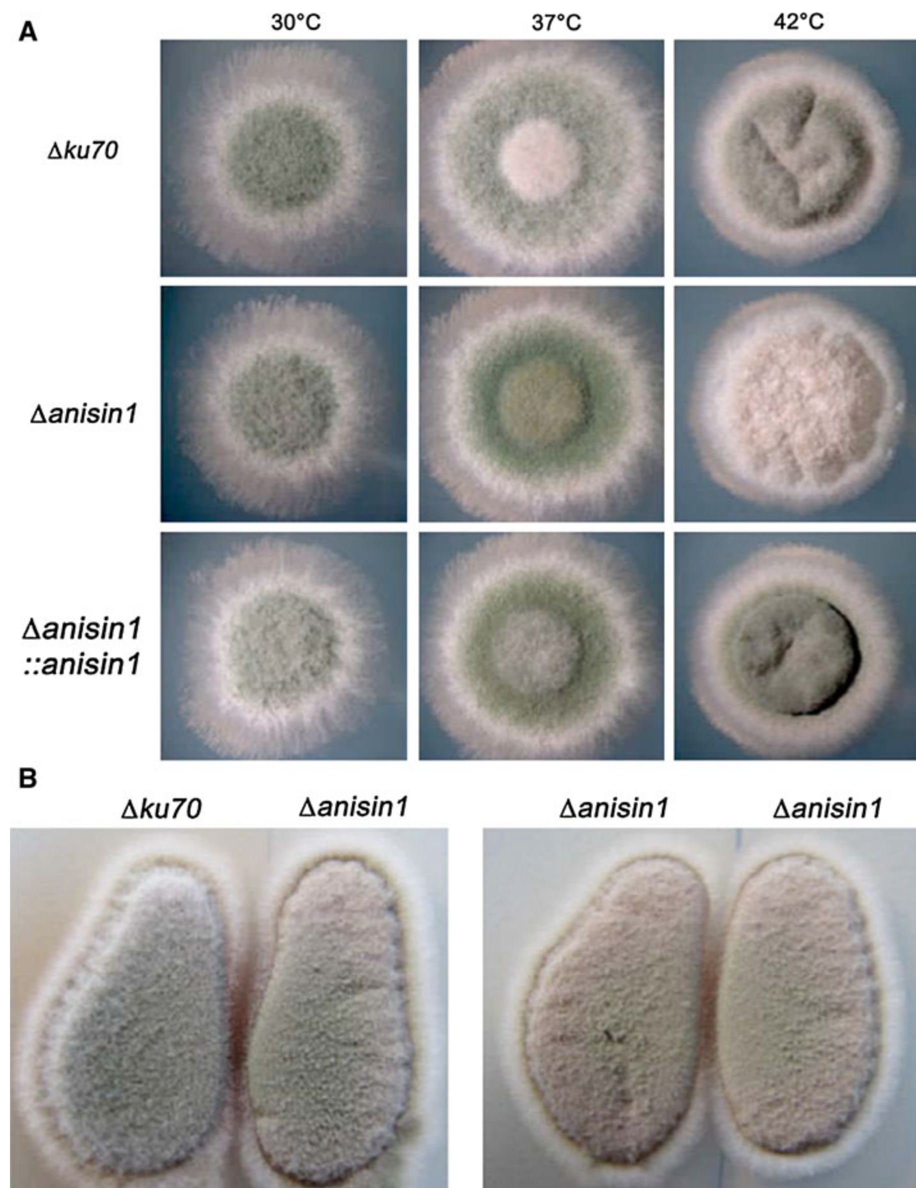

Fig. 2 Impact of *anisin1* deletion in asexual development of *A. nidulans*. **a** Surface cultures of *A. nidulans* reference strain Δ*ku70*, the *anisin1* deletion strain and Δ*anisin1::anisin1* complemented strain. 2×10^3 conidia were point inoculated on solid MM and incubated for 48 h at 30, 37 and 42°C, respectively. **b** Co-cultivation of *A. nidulans* Δ*ku70*/Δ*anisin1* (*left panel*) and Δ*anisin1*/Δ*anisin1* (*right panel*). 5×10^5 conidia were point inoculated on solid MM and incubated for 40 h at 42°C

Table 1 The conidial number of *A. nidulans* Δ*ku70*, the deletion mutant Δ*anisin1* and the complemented strain Δ*anisin1*::*anisin1* that were point inoculated (2×10^3 conidia) on solid MM agar plates

Time (h)	Temperature (°C)	Number of conidia[a]		
		Δ*ku70*	Δ*anisin1*	Δ*anisin1::anisin1*
48	30	$2.6 \times 10^5 \pm 1.8 \times 10^4$	$2.3 \times 10^5 \pm 1.8 \times 10^4$ (−10%)	$2.6 \times 10^5 \pm 1.3 \times 10^4$ (0%)
	37	$4.2 \times 10^5 \pm 3.6 \times 10^4$	$3.6 \times 10^5 \pm 1.8 \times 10^4$ (−15%)	$4.2 \times 10^5 \pm 1.9 \times 10^4$ (0%)
	42	$6.8 \times 10^4 \pm 4.3 \times 10^3$	$4.7 \times 10^3 \pm 4.4 \times 10^2$ (−93%)	$6.5 \times 10^4 \pm 2.1 \times 10^3$ (−5%)
72	30	$6.5 \times 10^5 \pm 1.3 \times 10^4$	$6.4 \times 10^5 \pm 1.6 \times 10^4$ (−1%)	$6.4 \times 10^5 \pm 2.4 \times 10^4$ (−2%)
	37	$1.2 \times 10^6 \pm 2.1 \times 10^4$	$9.5 \times 10^5 \pm 3.7 \times 10^4$ (−21%)	$1.1 \times 10^6 \pm 2.1 \times 10^4$ (−9%)
	42	$3.8 \times 10^5 \pm 1.1 \times 10^4$	$1.3 \times 10^5 \pm 8.5 \times 10^3$ (−65%)	$3.2 \times 10^5 \pm 6.3 \times 10^3$ (−16%)

[a] The percentage (%) of the relative change in conidiation efficiency of the mutants compared to the reference strain (=100%) is shown in brackets

according to the respective loading controls (18S and 26S rRNA) and then the values of the Δ*anisin1* mutant were correlated with the Δ*ku70* strain. Strikingly, at 42°C the *brlA* expression was severely retarded in the Δ*anisin1* mutant and the amount of *brlA* mRNA was reduced by 70% after 24 h. At later time points, *brlA* expression increased (−18% after 36 h) and finally reached +87% after 48 h compared to the control strain (Fig. 3; Table 2). This may reflect the amelioration in the production of conidia in the Δ*anisin1* mutant with later incubation times (Table 1). Therefore, we conclude that the defect in mitospore production of Δ*anisin1* at 42°C results from a delayed and reduced expression of *brlA*.

It is known that the intracellular level of reactive oxygen species (ROS) regulates most diverse cellular functions in *Aspergillus* sp., among others also hyphal differentiation and mitospore production (Hansberg and Aguirre 1990; Aguirre et al. 2005; Reverberi et al. 2008). As such, hyperoxidant conditions trigger microbial differentiation (Kawasaki et al. 1997). The increase in *catB* expression, which is one of the major scavengers against H_2O_2, is indicative for elevated endogenous ROS levels during cell differentiation as well as for the response to oxidative stress–inducing agents such as H_2O_2 (Hansberg et al. 1993; Kawasaki et al. 1997). Therefore, it is crucial for microorganisms, such as *A. nidulans*, that the oxidative stress response is tightly regulated (Pócsi et al. 2005; Miskei et al. 2009; Balázs et al. 2010).

To answer the question whether Anisin1 function may be interconnected with oxidative stress response in asexual development, we investigated the expression pattern of *catB* in the surface cultures of Δ*ku70* and Δ*anisin1* (Fig. 3). At 30°C, the *catB* expression pattern of Δ*anisin1* resembled that of Δ*ku70*. Instead, the intensity of the *catB* signals was moderately, but consistently higher in the Δ*anisin1* strain grown at 37 and 42°C compared to the reference strain (Fig. 3; Table 2). At 37°C, the *catB* expression increased by 66% after 24 h, 84% after 36 h and 71% after 48 h in the mutant strain compared to the control (Δ*ku70*). At 42°C, the amount of *catB* mRNA increased by 78% after 24 h, 44% after 36 h and 182% after 48 h in the Δ*anisin1* strain compared to the Δ*ku70*.

This indicates that depletion of *anisin1* not only leads to the deregulation of *brlA*, but also moderately triggers *catB*

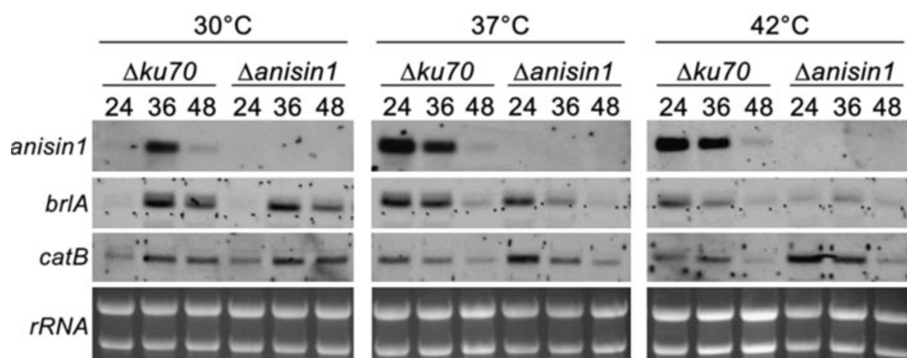

Fig. 3 Northern blot analysis of the expression of *anisin1*, *brlA* and *catB* in surface cultures of the *A. nidulans* reference strain Δ*ku70* and the Δ*anisin1* mutant, respectively, after 24, 36 and 48 h incubation at 30, 37 and 42°C. 10 μg of total RNA was loaded into each well, blotted and hybridized with the respective gene-specific digoxigenin-labeled probes. Ethidiumbromide-stained 26S and 18S rRNA are shown as loading controls

Table 2 Quantification of signal intensities on Northern blots of *brlA* and *catB* mRNA derived from the Δ*anisin1* mutant compared to Δ*ku70* (=100%)

Temperature (°C)	Incubation time (h)	*brlA* (%)	*catB* (%)
37	24	−40	+66
	36	−63	+84
	48	−60	+71
42	24	−70	+78
	36	−18	+44
	48	+87	+182

The ratio of gene-target expression to 26S and 18S rRNA (internal standards) was calculated, and the corrected intensities were compared between the mutant and the reference strain using the Image Quant 5.2 Software (Molecular Dynamics)

expression in developing *A. nidulans*. Possibly, the developmental response to hyperoxidant conditions at elevated temperatures is impaired in the *anisin1*-depleted strain, and a ROS threshold higher than in the Δ*ku70* is needed for the onset of differentiation. Importantly, except for the Δ*anisin1* mutant cultivated at 42°C, the *catB* transcription pattern correlated with that of *brlA* and *anisin1* in the Δ*ku70* and with that of *brlA* in the Δ*anisin1* at the growth temperatures of 30 and 37°C, respectively. Thus, our observations further support the role of *anisin1* in the developmental regulation of *A. nidulans*.

Finally, to confirm or dismiss the hypothesis that the Δ*anisin1* mutant suffers from an impaired oxidative stress response, we tested the sensitivity of Δ*anisin1* to 20–100 mM H_2O_2 (hydroxyl radical generator), 1–10 mM menadione and 0.1–0.5 M paraquat (both superoxide radical generators) by agar diffusion tests. No difference in the susceptibility between the *anisin1* deletion mutant and the reference strain Δ*ku70* was detectable (data not shown). This result let us conclude that Anisin1 is not primarily involved in oxidative stress response such as the detoxification of oxidative radicals.

anisin1 expression is deregulated in a Δ*srrA* mutant

To investigate in depth the role of *anisin1* in asexual development, we monitored the *anisin1* transcription in an *A. nidulans* mutant defective in SrrA function compared to the wild-type strain BPU1. SrrA is a Skn7-type response regulator (RR) in the His-Asp phosphorelay signaling pathway, which supports the ability of growth and survival under unfavorable environmental conditions. In the last years, several studies contributed to the characterization of some of the major players of this pathway in yeasts and filamentous fungi (Maeda et al. 1994; Posas et al. 1996; Hagiwara et al. 2007a, b; Izumitsu et al. 2007; Vargas-Pérez et al. 2007; Ruprich-Robert et al. 2008; Zhang et al. 2010). In *A. nidulans*, one of several histidine kinases (HK), namely NikA, senses environmental signals and transmits it to RRs. The transfer of phosphoryl groups (phosphorelay system) renders the RRs functional, generally as transcriptional regulators (SrrA, NapA) or as intermediate regulators (SskA) of mitogen-activated kinases (Mpk), like HogA, which in turn activate specific transcriptional regulators (Asano et al. 2007; Vargas-Pérez et al. 2007; Hagiwara et al. 2009). Apart from osmotic adaptation, fungicide inhibition and cell wall resistance, SrrA has been implicated in asexual development and oxidative stress response (Hagiwara et al. 2007a; Vargas-Pérez et al. 2007). Notably, the Δ*srrA* mutant produces less conidia compared to the wild-type strain and is defective in *catB* induction. Thus, the detoxification of ROS, especially H_2O_2, is impaired (Hagiwara et al. 2007a; Vargas-Pérez et al. 2007). Due to these phenotypical features, the Δ*srrA* mutant represented a valuable *A. nidulans* strain to study the function of *anisin1*.

First, we performed Northern blot analysis to monitor the transcription levels of *anisin1*, *brlA* and *catB* for 24 h in synchronized surface cultures of the Δ*srrA* mutant compared to the wild-type strain (Fig. 4). In the wild-type strain, *anisin1* transcripts could be detected 6–24 h after the hyphae were exposed to the air (Fig. 4). This correlated with the expression of *brlA* and the induction of *catB* transcription. In contrast, *anisin1*, *brlA* and *catB* expressions were deregulated in the Δ*srrA* mutant (Fig. 4). The *anisin1* gene was derepressed already in the 18 h old submers preculture before the hyphae were exposed to the air (0 h), and the transcription decreased after 12 h of synchronization. Only low amounts of *brlA* mRNA could be detected 6 h after synchronization, and the induction of *brlA* expression was limited to a very short time (12 h after synchronization), which reflects the reported conidiation defect of this *A. nidulans* mutant (Hagiwara et al. 2007a; Vargas-Pérez et al. 2007). Importantly, this result points toward a BrlA-independent induction of *anisin1* transcription.

The expression of *catB*, however, was not triggered by the exposure of the Δ*srrA* mutant to the air. The *catB* expression was similarly expressed at the time points 0 and 24 h and was even slightly decreased at the time points 6 and 12 h (Fig. 4). This indicates that Δ*srrA* is unable to adequately respond to the modulation of endogenously generated H_2O_2 levels when exposed to the air, and this might contribute at least in part to the defect in mitospore production.

Vargas-Pérez et al. (2007) documented that mild hyperosmotic conditions rescued the conidiation defect of the Δ*srrA* mutant possibly by channeling oxidative stress toward SskA activation. Therefore, we tested the effect of 0.6 M sucrose on the expression of *anisin1*, *brlA* and *catB* in the Δ*srrA* mutant and used the wild type as a control

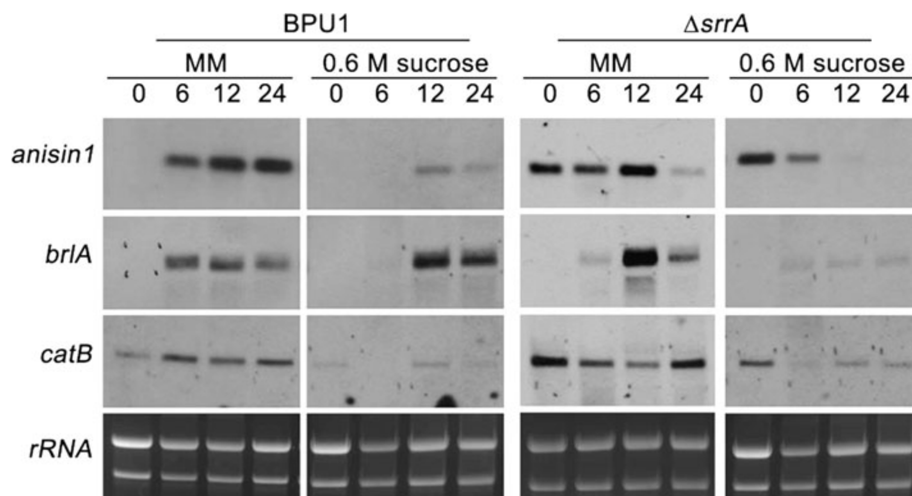

Fig. 4 Northern blot analysis of the expression of *anisin1*, *brlA* and *catB* in synchronized surface cultures of *A. nidulans* wild-type BPU1 and Δ*srrA* grown at 37°C under isotonic and hypertonic (0.6 M sucrose) conditions. Total RNA was extracted at the indicated time points (0, 6, 12 and 24 h) with time point 0 h referring to the liquid preculture before exposure to the air. 5 μg of total RNA was loaded into each well, blotted and hybridized with the respective gene-specific digoxigenin-labeled probes. Ethidiumbromide-stained 26S and 18S rRNA are shown as loading controls

(Fig. 4). The supplementation of the growth medium with 0.6 M sucrose repressed *catB* transcription in the wild-type strain, and the transcription of *anisin1* and *brlA* was delayed by 6 h compared to normal growth conditions. Furthermore, the amount of *anisin1* mRNA was significantly less under mild hyperosmotic growth conditions. The repression of *catB* transcription in the wild-type strain implies that high osmolarity lowers the oxidative stress burden of the developing cells.

Hyperosmotic growth conditions also repressed *catB* transcription in the Δ*srrA* mutant when compared to normal growth conditions. However, the expression of *anisin1* decreased already after 6 h of exposure to the air, and minimal amounts of *brlA* mRNA were detectable over the time course investigated. This result points toward a severely impaired asexual development of the Δ*srrA* at least within the first 24 h. Thus, a remediation of the sporulation defect of this mutant by high osmolarity could not be observed at the molecular level within 24 h after exposition to the air and contrasts to the report of Vargas-Pérez et al. (2007). This discrepancy might be explained by the different experimental setup that we applied in our study: we used synchronized surface cultures and monitored the gene expression over 24 h whereas Vargas-Pérez and coworkers analyzed the number of conidia of point inoculated surface cultures incubated for 6 days (Vargas-Pérez et al. 2007). It is conceivable to assume that the formation of conidia in the Δ*srrA* strain ameliorates during successive developmental generations incubated for longer times on hyperosmotic medium.

Further evidence that hyperosmotic growth conditions do not trigger *anisin1* expression in a submers *A. nidulans* wild-type culture is provided by the transcriptome analysis for the *A. nidulans* HogA Mpk signaling pathway in response to osmotic stress that has been recently published by Hagiwara et al. (2009) (Gene Expression Omnibus (GEO) from NCBI, accession number GSE16566; data not shown).

Next, we wanted to investigate in more detail the deregulation of *anisin1* in a Δ*srrA* submers culture. To this end, we compared the *anisin1* expression pattern of the Δ*srrA* and other mutants of the His-Asp phosphorelay pathway. In contrast to the situation in the Δ*srrA* mutant, *anisin1* was repressed in the wild-type strain and in the mutants Δ*sskA* and Δ*hogA* (Fig. 5). This was also true for the deletion mutants Δ*nikA* and Δ*napA* (data not shown). The derepression of *anisin1* in Δ*srrA* was even more pronounced under H_2O_2 (20 mM for 2 h) stress (Fig. 5). To monitor reduced CatB function in the Δ*srrA* strain, we determined the *catB* expression as well. H_2O_2 triggered *catB* transcription in the wild type and in the Δ*sskA* and Δ*hogA* mutants, but not in the Δ*srrA* mutant when compared to the respective untreated controls (Fig. 5). This clearly shows that CatB-mediated detoxification of H_2O_2 was impaired in this strain. These data further indicate that *anisin1* expression is derepressed in a Δ*srrA* mutant that suffers from defective H_2O_2 detoxification. The ROS burden of the cell increases even more upon H_2O_2 challenge, which is accompanied by a substantial increase in *anisin1* expression.

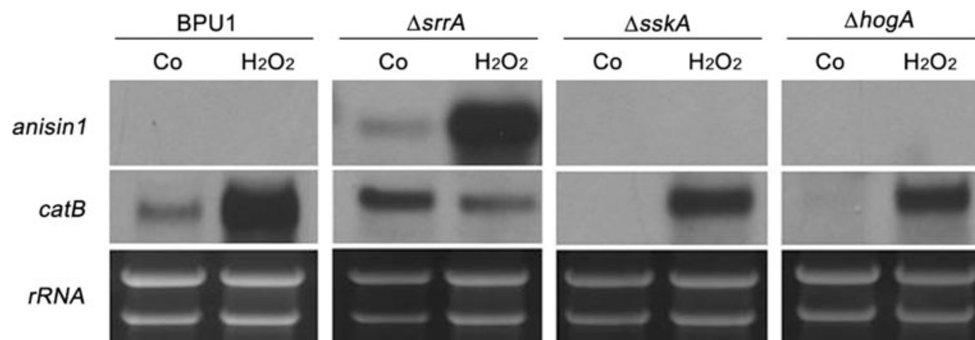

Fig. 5 Northern blot analysis of the expression of *anisin1* and *catB* in liquid cultures of *A. nidulans* wild-type BPU1 and single mutants of the His-Asp phosphorelay pathway Δ*srrA*, Δ*sskA*, Δ*hogA* challenged with 20 mM H_2O_2 for 2 h. Controls (Co) were left untreated. 5 µg of total RNA was loaded into each well, blotted and hybridized with the respective gene-specific digoxigenin-labeled probes. Ethidiumbromide-stained 26S and 18S rRNA are shown as loading controls

Conclusion

Our results indicate that *anisin1* codes for a secreted protein that supports optimal conidiation of *A. nidulans*, and we propose that Anisin1 directly or indirectly modulates the expression of *brlA* and/or the activity of BrlA, possibly by interacting with other factors that are involved in developmental regulation of *A. nidulans*. These factors could be, for example, StuA, AbaA (Adams et al. 1998) or other so far unidentified players. This assumption, however, remains to be evidenced at the molecular level, and it is crucial to identify the interacting partner of Anisin1.

Importantly, *anisin1* is not directly involved in the detoxification of ROS, because (1) depletion of *anisin1* did not increase the susceptibility of *A. nidulans* to externally applied ROS-inducing agents and (2) *anisin1* expression was not triggered by externally applied H_2O_2 in *A. nidulans* wild type. In a Δ*srrA* mutant, however, *anisin1* was derepressed under standard growth conditions and its expression was strongly induced by externally applied H_2O_2. Further studies are needed to address the question whether SrrA directly or rather indirectly regulates *anisin1* expression and which signal transduction pathways are involved in *anisin1* regulation.

For completion, it has to be noted that no expression of the *anisin1*-related *anisin2/atesin3* gene could be detected neither in the Δ*anisin1* mutant and the respective reference strain Δ*ku70* nor in the mutants of the His-Asp phosphorelay signaling pathway and the wild-type strain BPU1 under all conditions tested (data not shown). This excludes a redundant function of this gene and indicates that it plays a different role than *anisin1*.

The existence of various endogenous secreted molecules that regulate growth and conidiation point to the possibility that autoinducer-mediated mechanisms are widespread among filamentous fungi (Lee and Adams 1994; Roncal et al. 2002; Tsitsigiannis and Keller 2007). Based on our results, some conditional parallels between the effect of Anisin1 and other modulators of growth and development can be drawn. For example, the cysteine-rich secreted antifungal protein PAF of *P. chrysogenum* helps the producing mold to adjust to variable environmental conditions by balancing asexual development (Hegedüs et al. 2011a, b). This function, in addition to its antifungal activity, provides *P. chrysogenum* an ecological advantage over concurring organisms. Work is in progress, however, to verify a putative antimicrobial function of Anisin1.

Interestingly, defensins of higher eukaryotes are central players in the modulation of the innate immune system and in stress signaling. Both functions have been proposed to be even more relevant than their antimicrobial activities (Lowenberger 2001). Notably, cysteine-rich small molecular proteins are perfect targets for oxidative or reductive protein transformation resulting in a conformational change that could influence and modulate their activity and explain their variable function in response to environmental stimuli, such as hyperoxidant states or hypoxic conditions (Hansberg and Aguirre 1990; Aguirre et al. 2005; Batta et al. 2009). In this respect, our results let us propose that *anisin1* plays a role in sensing intracellular H_2O_2, which arises either in response to developmental signals or due to defective detoxification. The presence of putative regulatory elements, like STRE, in the *anisin1* promoter could further indicate that *anisin1* is a stress-responsive gene. This, however, needs to be clarified in further experiments.

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