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Could the transcription factor AtnN coordinating the aspercryptin secondary metabolite gene cluster in *Aspergillus nidulans* be a global regulator?

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ABSTRACT

Products of dormant secondary metabolite gene clusters of fungal genomes can be exploited for medical purposes as bioactive agents. These clusters can be switched on under oxidative stress and may endow fungi with a versatile chemical armory in a competitive niche. In *Aspergillus nidulans,* the aspercryptin gene cluster, including the synthase [*atnA* (AN7884)] and its transcription factor (*atnN*), was activated under menadione sodium bisulfite (MSB) treatment. In this study, we generated and phenotypically examined the gene deletion and overexpression mutants of *atnN* and studied the secondary metabolite production of the mutants. Overexpression of *atnN* significantly reduced the colony growth of surface cultures compared to the control. The *ΔatnN* gene deletion strain showed higher sensitivity to *tert*-butyl hydroperoxide (*t*BOOH), while the *atnN*OE strain was more resistant to MSB, Congo Red, and sorbitol. Interestingly, deletion of *atnN* decreased cleistothecia formation of *A. nidulans*. Manipulation of *atnN* affected the synthesis of several secondary metabolites, for example, the siderophore production of *A. nidulans*. The extracellular triacetylfusarinine C (TAFC) production decreased, while the intracellular ferricrocin (FC) concentration of the cultures increased in the *atnN*OE mutant cultivating *A. nidulans* in a complex medium containing 1 % mycological peptone and 2 % maltose. In Czapek-Dox Broth medium, increased asperthecin production was observed in the *ΔatnN* mutant. The mycotoxin sterigmatocystin synthesis elevated in the *ΔatnN* mutant, while reduced in the *atnN*OE mutant on minimal medium. Our study supports previous observations that secondary metabolite production is coordinated in a complex way, and the linkage of stress response, sexual reproduction, and secondary metabolite production can be governed by several transcription factors.

1. Introduction

Fungi seem to have unlimited production of bioactive secondary metabolites, including antibiotics like penicillin, immunosuppressants like cyclosporine, antimycotics such as griseofulvin or echinocandins, and cholesterol-lowering drugs like lovastatin (Yaegashi et al., 2014). Whole genome sequencing and annotation projects of fungi have recently elucidated the plethora of secondary metabolite gene clusters, including silent, further exploitable gene clusters (Chiang et al., 2008, 2010; Giles et al., 2011; Ahuja et al., 2012; Inglis et al., 2013; Yaegashi et al., 2014; Keller, 2019). These dormant clusters can be activated under stressful conditions and armor fungi with useful chemicals in their natural competitive habitat (Keller et al., 2005; Yaegashi et al., 2014). It is well-known that secondary metabolite production can be upregulated by stress, *e.g.*, lipid peroxidation under laboratory conditions (Reverberi et al., 2010; Hong et al., 2013; Amare and Keller, 2014). For example, in *Aspergillus nidulans*, numerous secondary metabolite gene clusters were activated under exposure to oxidative [menadione sodium bisulfite

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(MSB), H_2O_2, *t*-butylhydroperoxide (*t*BOOH), diamide] or osmotic (NaCl) stress (Emri et al., 2015). Among these clusters, the AN7884 cluster was found to be up-regulated during MSB stress, which was identified later as an aspercryptin cluster by several research groups (Chiang et al., 2016; Henke et al., 2016). Aspercryptin was first isolated from a genetically dereplicated strain, in which eight of the most abundantly expressed secondary metabolite gene clusters, including sterigmatocystin (Brown et al., 1996), the emericellamides (Chiang et al., 2008), asperfuranone (Chiang et al., 2009), monodictyphenone (Chiang et al., 2010), terrequinone (Bok et al., 2006), F9775A and B (Sanchez et al., 2010), asperthecin (Szewczyk et al., 2008), and both portions of the split cluster that makes austinol and dehydroaustinol (Lo et al., 2012), were deleted. Aspercryptin is produced from two different secondary metabolite gene clusters, the cichorine and aspercryptin clusters separated physically in the genome of *A. nidulans*. Interestingly, the cichorine cluster encodes a polyketide synthase (PKS), while the aspercryptin cluster contains a core biosynthetic gene for non-ribosomal peptide synthetase (NRPS) (Chiang et al., 2016). The genome of *A. nidulans* accommodates twelve NRPS genes, including one in the aspercryptin cluster, and some of the well-known biomedical agents, including penicillin and cyclosporine, are also the products of NRPS gene clusters (von Dohren, 2009). Secondary metabolite gene clusters can also be activated by the inhibition of histone deacetylases. The aspercryptin gene cluster could be upregulated by up to 90-fold applying this strategy (Henke et al., 2016). In this study, we deleted and overexpressed the Zn(2)-Cys(6) binuclear cluster domain transcription factor *atnN* (locus ID: AN7872) coordinating the aspercryptin cluster expression (Inglis et al., 2013). Furthermore, we studied the stress tolerance, sexual development as well as secondary metabolite production profile of the mutants.

2. Materials and methods

2.1. Strains, culture media, and production of conidia

The following *A. nidulans* strains were used in our study: rJMP1.59 (*pyrG89*; *pyroA4*; *veA*$^+$), TNJ36.1 (*pyrG89; pyroA4; pyrG*$^+$*; veA*$^+$), THS30.3 (*pyrG89*, *AfupyrG*$^+$; *pyroA*$^+$; veA$^+$), *ΔatnN* (*pyrG89*; *ΔatnN*::*AfupyrG*$^+$; *pyroA*$^+$; *veA*$^+$) and *atnN*OE (*pyroA4*; *niiA(p)::atnN::pyroA3/4*; *veA*$^+$). All strains were maintained on Barratt's nitrate minimal medium (NMM) and supplemented with 0.05 mg/l pyridoxine, if necessary (Barratt et al., 1965). Conidia harvested from 6-day-old surface cultures incubated at 37 °C were used in all further experiments (Balázs et al., 2010).

2.2. Construction of *ΔatnN* gene deletion and *atnN*OE overexpression strains

Gene deletion mutants were constructed by the Double-Joint PCR method of Yu et al. (2004) and Leiter et al. (2016) with primers listed in Table S1. The amplified deletion cassettes were used to transform the rJMP1.59 strain using the Vinotaste Pro lysing enzyme (Szewczyk et al., 2006). Single-copy transformants were selected after PCR analysis (Yu et al., 2004). To generate overexpression mutants, ORF was amplified with the primers presented in Table S1. The amplicon was digested with restriction enzymes, as indicated in Table S1, and ligated between the *niiA* promoter and the *trpC* terminator in pHS11 (Leiter et al., 2016). Overexpression of the strains was confirmed by the rRTqPCR method (Table S1) (Kocsis et al., 2022).

2.3. Stress sensitivity studies

To study the stress sensitivity of the *ΔatnN* and *atnN*OE mutant strains, the agar plate assays of Balázs et al. (2010) were applied. The following stress-generating agents were tested -oxidative stress: 2.0 mM diamide (causing GSH/GSSG redox imbalance), 0.08 mM MSB (elevating intracellular superoxide level), 0.8 mM *t*BOOH (triggering lipid peroxidation), (Pócsi et al., 2005; Emri et al. 1997); heavy metal stress: 300 μM cadmium chloride; cell wall integrity stress: 75 μg/ml Congo Red; osmotic stress: 1.5 M NaCl and 2.0 M sorbitol. Plates were point-inoculated with 5 μl freshly made conidia suspension (2×10^7 conidia/ml) and were incubated at 37 °C for 5 days (Balázs et al., 2010). Diameters of the colonies were measured and used for the characterization of the stress sensitivities of the strains.

2.4. Sexual development

To induce cleistothecium formation, 6-day-old conidia were spread in agar at 10^5 conidia/plate and incubated at 37 °C. After 24 h, and plates were completely sealed with Parafilm. Samples were taken with a cork borer after 14 days of incubation, and cleistothecia/cm^2 was determined under a dissection microscope (Leiter et al., 2016).

2.5. Analysis of secondary metabolite production

To analyze the secondary metabolite production of the mutants, different culture media and conditions were applied. We tested the secondary metabolite production in a shaking culture containing NMM supplemented with 2 % maltose and 1 % peptone (supplemented with 0.05 mg/l pyridoxine if necessary) at 25 °C, at 220 rpm for 3 days inoculated with 5×10^7/100 ml conidiospores. In addition, static culture with Czapek-Dox Broth medium (3 g/l $NaNO_3$, 0.5 g/l KCl, 0.5 g/l $MgSO_4x7H_2O$, 1 g/l KH_2PO_4, 30 g/l sucrose and 1 ml/l 1000X TES pH = 4,8) was inoculated at 25 °C for 14 days in the dark with 750×10^6/150 ml conidia (Sanchez et al., 2010) as well as on NMM point-inoculated surface culture (Kocsis et al., 2022).

Siderophore production was determined according to Szigeti et al. (2014) from shaking cultures containing NMM supplemented with 2 % maltose and 1 % peptone (supplemented with 0.05 mg/l pyridoxine if necessary) by HPLC. Briefly, triacetylfusarinine C (TAFC) was determined from culture supernatants supplemented with 1 g/l $FeCl_3$, while ferricrocin (FC) was extracted from freeze-dried mycelia with distilled water. After centrifugation (5000×g, 10 min, 4 °C), the supernatant was supplemented with 1 g/l $FeCl_3$ as described above, and these extracellular and intracellular iron-treated samples were used for HPLC analyses. In HPLC measurements, 10 μl aliquots of the samples were injected into the reverse phase column (SupelcosilTM LC18, 150 × 4.6 mm, 5 μm), which was eluted with acetonitrile: 0.1 % TFA water gradient (starting at 6 % MeCN and ending at 40 % MeCN after 20 min and then washed for 10 min with MeCN) as the mobile phase at a flow rate of 1 ml/min at 25 °C using a Hewlett–Packard 1090 Series II liquid chromatograph also equipped with a diode array detector and an automatic sampler. The effluent was monitored at l = 435 nm, the adsorption maximum of FC and TAFC, and the signals were analyzed by the ChemStation software. The siderophore contents of the samples were calculated using standard curves and expressed as g/l culture medium (TAFC) or mg/g mycelium dry weight (FC).

Asperthecin concentration was determined from the freeze-dried and methanol-extracted mycelial samples cultivated in the static Czapek-Dox Broth medium by HPLC and detected by high-resolution Orbitrap mass spectrometry (HR-MS). The HPLC separations were performed on a Kinetex C18 column (75 × 3 mm; 2.6 μm) (Phenomenex, USA). Eluents: eluent A, 0.1 % v/v formic acid, eluent B, acetonitrile: 0.1 % v/v formic acid (80:20, v/v). Gradient: linear, 0.0 min, 20 % B; 12.0 min, 90 % B; flow rate: 0.3 mL/min; temperature: 25 °C; injected volume: 5.0 μL. The ESI source was operated in positive and negative ionization mode (switching mode). Fragmentations were performed by data-independent acquisition (DIA) method using isolation widths of 100–300 *m/z*, 295–500 *m/z*, 495–700 *m/z*, and 695–800 *m/z* and collision energy of 15, 30 and 45 eV. Operation parameters were optimized automatically by the built-in software as follows: spray voltage, 3500 V (+); capillary temperature 256 °C; sheath-, auxiliary- and spare-gases (N_2): 47.50, 11.25 and 2.25 arbitrary units, respectively. The resolutions of the full scans and MS/MS scans were 70,000 and 35,000, respectively. Full MS scanning range: 100–1000 *m/z* units. UV spectra were recorded between 250 and 600 nm, and UV chromatograms were plotted as summed signal intensities measured in this wavelength range.

Sterigmatocystin yield was measured according to Kocsis et al. (2022). Briefly, a 2 cm^2 agar plug was removed from each 5 d NMM (supplemented with 0.05 mg/l pyridoxine, if necessary) culture plate and extracted with 800 µl by 70 % (v/v) acetone and subjected to HPLC analysis. Briefly, aliquots of 10 µl samples were injected into the chromatographic system, which consisted of a Waters 2695 Separations Module equipped with a thermostable autosampler (5 °C) and column module (35 °C). UV detection was applied by a Waters 2996 photodiode array detector (254 nm). Separations were performed using an Agilent Zorbax SB-C18 (4.6 mm × 75 mm, 3.5 µm) column with 1 ml/min flow rate. Isocratic elution was used, where the mobile phase was methanol/acetonitrile/water 50/15/35 (v/v), respectively (Yin et al., 2013).

2.6. Statistical analysis of experimental data

All experiments were performed in three independent sets, and mean ± SD values were calculated and presented. Statistical significances were calculated using Student's *t*-test, and *p*-values less than 5 % were considered statistically significant.

3. Results and discussion

In previous studies, the AN7884 cluster was identified as an aspercryptin cluster (Chiang et al., 2016; Henke et al., 2016). In this study, we focused on mapping any changes in stress sensitivity, sexual development, and the secondary metabolite profile of *A. nidulans* by deletion and overexpression of the *atnN* transcription factor coordinating the aspercryptin secondary metabolite gene cluster.

Overexpression of *atnN* resulted in reduced colony growth of *A. nidulans* [6.56 ± 0.02 cm (colony growth of THS30.3) vs 5.5 ± 0.1 cm (colony growth of *atn*OE), p<1 %] (Fig. 1). Surprisingly, manipulation of the *atnN* transcription factor affected the stress sensitivity of *A. nidulans* (Fig. 1). *t*BOOH treatment increased the sensitivity of the *ΔatnN* mutant, while in the presence of MSB, Congo Red and sorbitol *atn*OE showed increased tolerance compared to the corresponding control (Fig. 1).

We also evaluated the fruiting body formation of the mutants. Deletion of *atnN* significantly decreased the development of cleistothecia compared to the control (Fig. 2).

Different culture media were applied to assess secondary metabolite production of the *ΔatnN* and *atnN*OE mutants. We detected siderophore production by HPLC analysis, cultivating the mutants in NMM supplemented with 2 % maltose and 1 % mycological peptone. Overexpression of *atnN* significantly decreased TAFC production of *A. nidulans* mycelia, but increased the intracellular FC yield, respectively (Fig. 3A and **B**). The siderophores TAFC and FC are the products of the NRPS *sidC* (Haas, 2003). FC biosynthesis is coordinated by the GATA transcription factor SREA in *A. nidulans* and lack of SREA increased FC production (Oberegger et al., 2001). Besides the central role of SREA, HapX bZIP type transcription factor is also crucial to governing siderophore production in *A. nidulans* (Schrettl et al., 2010). The role of these transcription factors is interplayed, because SreA inhibits the expression of *hapX* during iron abundance and HapX represses *sreA* during iron depletion (Hortschansky et al., 2007).

In a static culture of Czapek-Dox Broth medium, asperthecin was isolated at a higher concentration in the *ΔatnN* gene deletion mutant than the control (Fig. 4). The asperthecin cluster, containing an iterative type I polyketide synthase, had been identified by Szewczyk et al. (2008). The cluster-independent coordination of asperthecin production was also described by another research group (Szewczyk et al., 2008). Deletion of a gene encoding the small ubiquitin-like protein SUMO in *A. nidulans* caused a massive increase in the yield of asperthecin (Szewczyk et al., 2008). It is the main pigment of the ascospore of *A. nidulans* and protects ascospores from UV light (Palmer et al., 2021). Asperthecin gene cluster is active during sexual development, but not in the ascospore formation (Palmer et al., 2021). RsmA and NapA bZIP type transcription factors also modulate asperthecin production. Asperthecin concentration increased in the *OE*::*rsmA* mutant, but decreased in the *OE*::*napA* mutant (Yin et al., 2012).

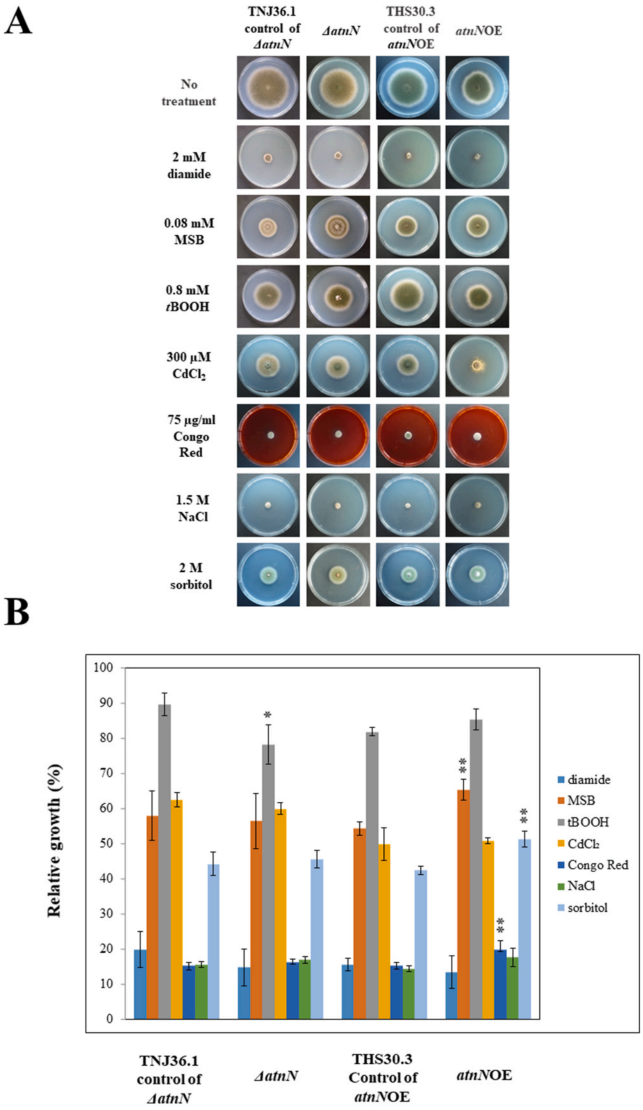

Fig. 1. Stress sensitivities of the TNJ36.1 (control) and the *ΔatnN* gene deletion mutant as well as THS30.3 (control) and *atnN*OE strains exposed to various types of stress. Plates were incubated at 37 °C for 5 days (**A**). Relative growth (%) was calculated by comparing the colony diameter of untreated and treated surface cultures within the same strain (**B**). Data are presented as mean ± SD values calculated from three independent experiments. Significant differences between controls and mutant cultures (*, p<5 %, **, p<1 %) are shown.

The mycotoxin sterigmatocystin synthesis was also altered in the mutants, as the deletion of *atnN* increased its production, while overexpression of *atnN* decreased the sterigmatocystin production of *A. nidulans* (Fig. 5).

The complex regulatory network of the secondary metabolite production of *A. nidulans* has been confirmed in several studies, for example, by the comparison of clinical isolates and A4 wild-type strain (Drott et al., 2020). One of the isolates was unable to produce sterigmatocystin, although its sterigmatocystin gene cluster proved to be intact according to genome sequencing. New clusters were also

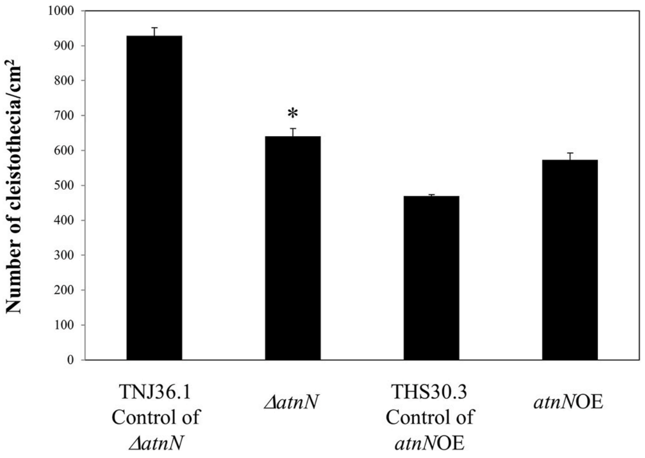

Fig. 2. Cleistothecia productions observed after 14 days of incubations. Data are presented as mean ± SD values calculated from three independent experiments. Significant differences between control and mutant culture pairs (*$p < 5$ %) are indicated.

identified in the clinical isolates, which cannot be found in the wild-type strain. These results suggest that even intraspecies variation can cause differences in the secondary metabolite production pattern of fungi (Drott et al., 2020).

Sterigmatocystin production can also be orchestrated by global regulators, including bZIP type transcription factors, *e.g.,* NapA and RsmA (Bákány et al., 2021; 2023). NapA is a *Saccharomyces cerevisiae* Yap1 ortholog and governs sterigmatocystin biosynthesis through the modulation of reactive species level (Bákány et al., 2021). RsmA (restorer of secondary metabolism A) is also a Yap-like protein and controls sterigmatocystin production directly. Both the overexpression and deletion of *rsmA* increased sterigmatocystin yield in *A. nidulans* (Bákány et al., 2021; Yin et al., 2013). *Schizosaccharomyces pombe* Atf1 bZIP ortholog AtfA also affects sterigmatocystin biosynthesis. Deletion of *atfA* in *A. nidulans* inhibits this mycotoxin production (Kocsis et al., 2022), similar to *Fusarium verticillioides* where no fumonisin mycotoxins were detected in the *ΔFvatfA* mutant (Szabó et al., 2020).

Linkage of stress response, sexual development, and sterigmatocystin production governed by bZIPs has been identified in *A. nidulans* (Yin et al., 2013). For example, overexpression of *rsmA* resulted in a high level of sterigmatocystin with less ascospore formation and with higher H_2O_2 sensitivity, while in *OE::napA* mutant inhibition of sexual development manifested in less cleistothecia with concomitant increase in

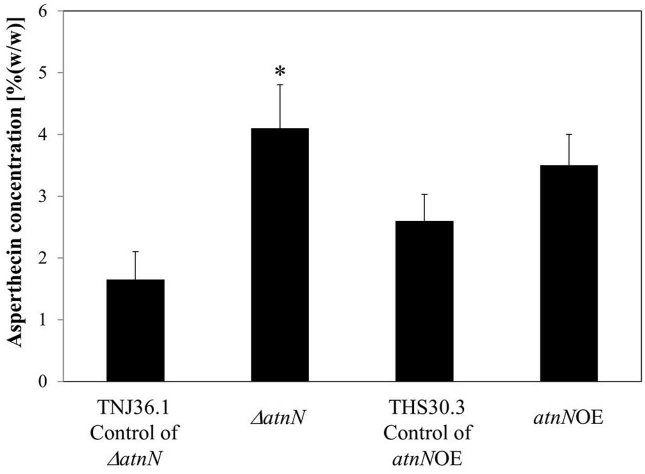

Fig. 4. Asperthecin production of the controls and mutant strains. Data are shown as mean ± SD values calculated from three independent experiments. Significant differences between control and mutant culture pairs (*$p < 5$ %) are indicated.

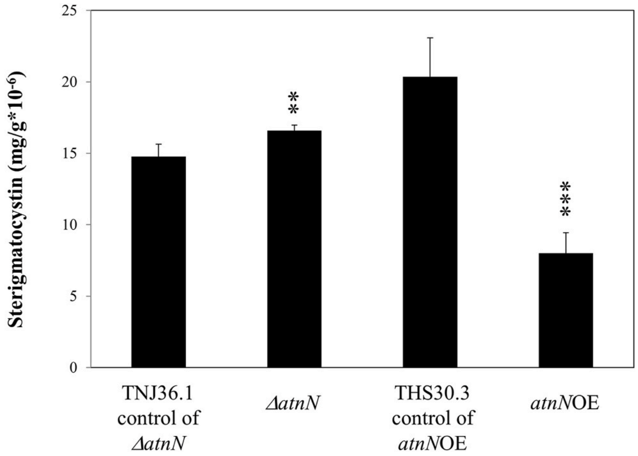

Fig. 5. Sterigmatocystin production of the controls and mutant strains. Data are presented as mean ± SD values calculated from three independent experiments. Significant differences between control and mutant cultures (**$p < 1$ %, ***$p < 0.1$ %) are indicated.

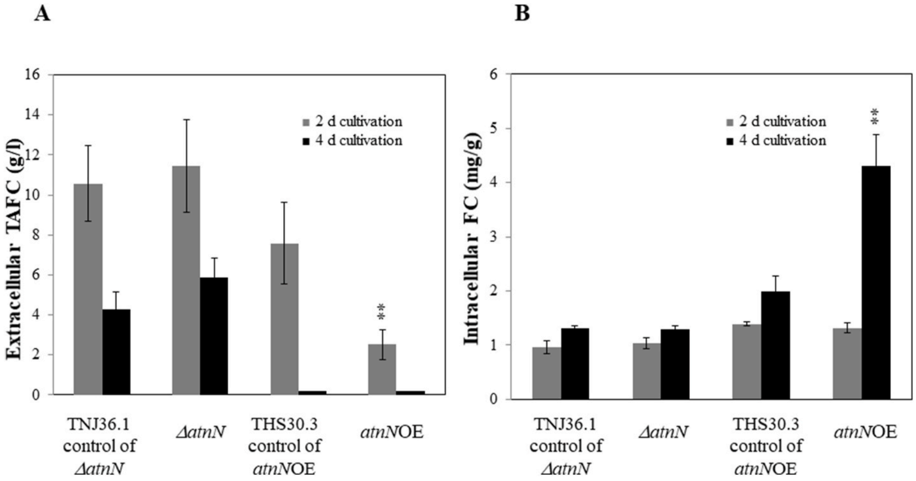

Fig. 3. Siderophore production (**A** TAFC and **B** FC) of the controls and *ΔatnN*, *atnN*OE mutants. Data are shown as mean ± SD values calculated from three independent experiments. Significant differences between control and mutant culture pairs (**$p < 1$ %) are also presented.

catalase activity (Yin et al., 2013; Bákány et al., 2021). Interestingly, AtnN as a local transcriptional regulator can affect stress response (Fig. 1) and sexual development (Fig. 2) as well as coordinate secondary metabolites including siderophore (Fig. 3), asperthecin (Fig. 4), and sterigmatocystin (Fig. 5) production. This is in line with previous observations that Zn(2)-Cys(6) binuclear cluster domain transcription factors like AtnN govern genes within other secondary metabolite gene clusters and several metabolic pathways (Keller, 2019).

4. Conclusion

Our study strengthens the evidence that the regulation of secondary metabolite gene clusters in *A. nidulans* is very diversified, which endows the fungus with maximum competitive advantage in harsh environments. Further studies should aim to elucidate whether the AtnN transcription factor is a global regulator and how it can fit into the complex regulatory network of secondary metabolite production. Our results can also contribute to the development of strategies focusing on the discovery of new bioactive agents and deeper understanding of the regulation of mycotoxin production in fungi.

CRediT authorship contribution statement

Beatrix Kocsis: Methodology, Investigation. **Imre Boldizsár:** Methodology. **Gábor M. Kovács:** Writing – review & editing, Validation. **Tibor Nagy:** Methodology. **Gyöngyi Gyémánt:** Methodology. **Kinga Csillag:** Methodology. **István Pócsi:** Writing – review & editing, Validation. **Éva Leiter:** Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2024.11.006.

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