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Evidence for an arginine-dependent route for the synthesis of NO in the model filamentous fungus *Aspergillus nidulans*

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Summary

Nitric oxide (NO) is a signalling molecule in eukaryotic and prokaryotic organisms. NO levels transiently boost upon induction of conidiation in *Aspergillus nidulans*. Only one pathway for NO synthesis involving nitrate reductase has been reported in filamentous fungi so far, but this does not satisfy all the NO produced in fungal cells. Here we provide evidence for at least one additional biosynthetic pathway in *A. nidulans* involving L-arginine or an intermediate metabolite as a substrate. Under certain growth conditions, the addition of L-arginine to liquid media elicited a burst of NO that was not dependent on any of the urea cycle genes. The NO levels were controlled by the metabolically available arginine, which was regulated by mobilization from the vacuoles and during development. *In vitro* assays with protein extracts and amino acid profiling strongly suggested the existence of an arginine-dependent NO pathway analogous to the mammalian NO synthase. Addition of polyamines induced NO synthesis, and mutations in the polyamine synthesis genes *puA* and *spdA* reduced the production of NO. In conclusion, here we report an additional pathway for the synthesis of NO in *A. nidulans* using urea cycle intermediates.

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Introduction

Nitric oxide (NO), an abundant short-lived radical, is a signalling molecule in all organisms from bacteria to mammals. Its signalling role has been extensively studied in mammals, in which it regulates many biological processes such as smooth muscle tone, platelet aggregation and adhesion, cell growth, apoptosis, neurotransmission, vasoconstriction, the reproductive system and sexual behaviour (Rosselli *et al*., 1998). In plants it regulates growth, development, photoperiod and flowering (He *et al*., 2004; Kwon *et al*., 2012). In microbes it is involved in processes such as the response to hypoxic conditions in bacteria (Seth *et al*., 2012) or in sporulation in the plasmodial slime mould (Golderer *et al*., 2001). In filamentous fungi, NO regulates several processes. For example, in *Aspergillus nidulans* NO regulates nitrogen metabolism and reproduction (Schinko *et al*., 2010; Schinko *et al*., 2013; Canovas *et al*., 2016; Marcos *et al*., 2016; Marcos *et al*., 2020), in *Neurospora crassa* NO also regulates conidiation (Pengkit *et al*., 2016), in *Magnaporthe oryzae* removal of NO delays germination and attenuates plant infection (Samalova *et al*., 2013). NO also plays a regulatory role in the establishment of plant–fungal mutualistic interaction, although the studies about this are still scarce (Martinez-Medina *et al*., 2019). NO contributes to the regulation of the active penetration by the plant pathogen and also to the recognition and host defence mechanisms (Jedelska *et al*., 2021). For example, in *Fusarium* it contributes to the transmission of the pre-contact signal from the plant and is required for pathogenesis (Ding *et al*., 2020). The pathogen itself protects its cells against excess of NO by activating NO-sensitive transcription factors that turn on NO oxidases (Jian *et al*., 2021). *A. nidulans* can undergo two developmental programs upon induction of reproduction, either the asexual (conidiation) or sexual (formation of cleistothecia) reproduction. The transition from vegetative growth to reproduction occurs as a response to specific signals, such as an air interphase (Adams *et al*., 1998; Etxebeste *et al*., 2010; Park and Yu, 2012; Ojeda-Lopez *et al*., 2018). After the induction, the balance between both reproduction programs is controlled by several

environmental signals (Rodriguez-Romero *et al*., 2010). NO boosts at the transition from vegetative growth to reproduction in *A*. *nidulans*. Later in the developmental process, NO levels also seem to contribute to the balance between asexual and sexual reproduction. Additionally, there is a cross-talk between light and NO to regulate the balance between asexual and sexual reproduction (Marcos *et al*., 2020).

NO can be synthesized by several biological pathways in living organisms, as well through non-enzymatic reactions under acidic conditions by disproportionation and reduction. Biologically it can be generated by nitric oxide synthases (NOS) using L-arginine as substrate (Zweier *et al*., 1999; Alderton *et al*., 2001; Gorren and Mayer, 2007; Gupta *et al*., 2011b). In mammals, NOS is responsible for the conversion of arginine and oxygen to citrulline and NO (Gorren and Mayer, 2007). In plants, the scenario is not that clear, and although it has been demonstrated that there is a route for NO synthesis that utilizes arginine as a substrate, the existence of an alleged NOS is still a matter of debate (Gupta *et al*., 2011a) and has only been clearly identified in the green alga *Ostreococcus* (Foresi *et al*., 2010; Jeandroz *et al*., 2016). In fungi, classical NOS are absent. Early work connected nitrate reductase (NR) activity to blue light-promoted conidiation in *N*. *crassa* (Klemm and Ninnemann, 1979), and later our laboratories have shown the contribution of the nitrate pathway to the synthesis of NO in *A*. *nidulans* (Marcos *et al*., 2016). However, NR mutants only reduce NO levels to around half of the wild-type level suggesting that additional enzymatic or non-enzymatic routes of NO generation exist in this fungus. Recent work in *Fusarium* also suggested that there are more routes for the synthesis of NO in addition to the NR pathway (Ding *et al*., 2020). These alternative routes remain elusive in filamentous fungi. Deletion of putative NOS genes in *M*. *oryzae* and *N*. *crassa* (Samalova *et al*., 2013; Pengkit *et al*., 2016) and in *A*. *nidulans* (our unpublished work) did not reduce NO synthesis, and mutants in the urea cycle did not affect NO synthesis in *M*. *oryzae* (Zhang *et al*., 2015). However, a recent report establishes connections between the NO-metabolizing flavohemoglobins, arginine and development (Marcos *et al*., 2020), suggesting that a NO synthesis pathway utilizing arginine as a precursor could be operative.

In this work, we found that alteration of the NO levels by growth on different nitrogen sources resulted in delayed and reduced conidiation. We investigated the origin of this NO as an additional route for the synthesis of NO involving metabolites of the urea cycle and polyamines. This route displays characteristics similar to the mammalian NOS.

Results

Growth on arginine reduces conidiation

In previous studies, we and others observed that NO homeostasis regulates the balance between asexual and sexual reproduction (Maier *et al*., 2001; Marcos *et al*., 2016; Marcos *et al*., 2020; Zhao *et al*., 2021). Generally, higher levels of NO promote cleistothecia formation whereas, at the same time, it represses conidiation. Consistently, increasing the NO levels with a chemical donor resulted in strongly reduced numbers of conidia. However, we also found that a transient increase of NO is associated with the onset of conidiation and that arginine produces a higher NO level compared to other nitrogen sources such as nitrate. Here we further explored the inhibitory effects of NO on conidiation and the connection with arginine. We employed an arginase mutant (Δ*agaA*) that is strongly impaired in metabolizing arginine. Conidiation levels were compared between the wild-type strain, a double mutant in the flavohemoglobins (Δ*fhbA* Δ*fhbB*) that has a reduced capacity for NO detoxification (Schinko *et al*., 2010; Marcos *et al*., 2016), a single mutant in the arginase (Δ*agaA*) and the triple mutant (Δ*fhbA* Δ*fhbB* Δ*agaA*). Growth on arginine provoked a reduction in conidia numbers compared to the growth on ammonium (Fig. 1A). This reduction was more notorious in the triple mutant Δ*fhbA* Δ*fhbB* Δ*agaA*, which can barely metabolize arginine and has reduced NO oxidation capacity. Furthermore, the consequences of increased arginine concentrations in the solid growth medium on conidiation were also evident at the level of colony morphology (Fig. 1B). Supplementation of solid media with 10 mM arginine produced a partially fluffy phenotype in the wild type strain and this effect was more drastic in the mutant lacking both flavohemoglobins (Δ*fhbA* Δ*fhbB*). Supplementation of the media with high concentrations (100 mM) of nitrate or ammonium had little effect (ammonium) or no effect (nitrate) on conidiation in both strains, suggesting that the effect produced by 10 mM arginine (which provides an equivalent to 40 mM of N) is not due to an excess of N in the media.

To test if the reduced conidiation is correlated to lower expression of conidiation-related genes and not only a consequence of reduced growth, we monitored expression of the hydrophobin *dewB* fused to red fluorescent protein *mrfp*, which is specifically expressed in conidia (Grunbacher *et al*., 2014), as a biomarker for conidiation, in parallel to colony growth on solid media. This method allows the parallel quantification of growth by absorbance measurement at 600 nm and reporter gene expression by fluorometry in actively growing colonies in a 96-well plate format on solid media (Canovas *et al*., 2017). Results shown in Suppl. Fig. S1 confirmed that growth of the wild type was equivalent on all tested nitrogen

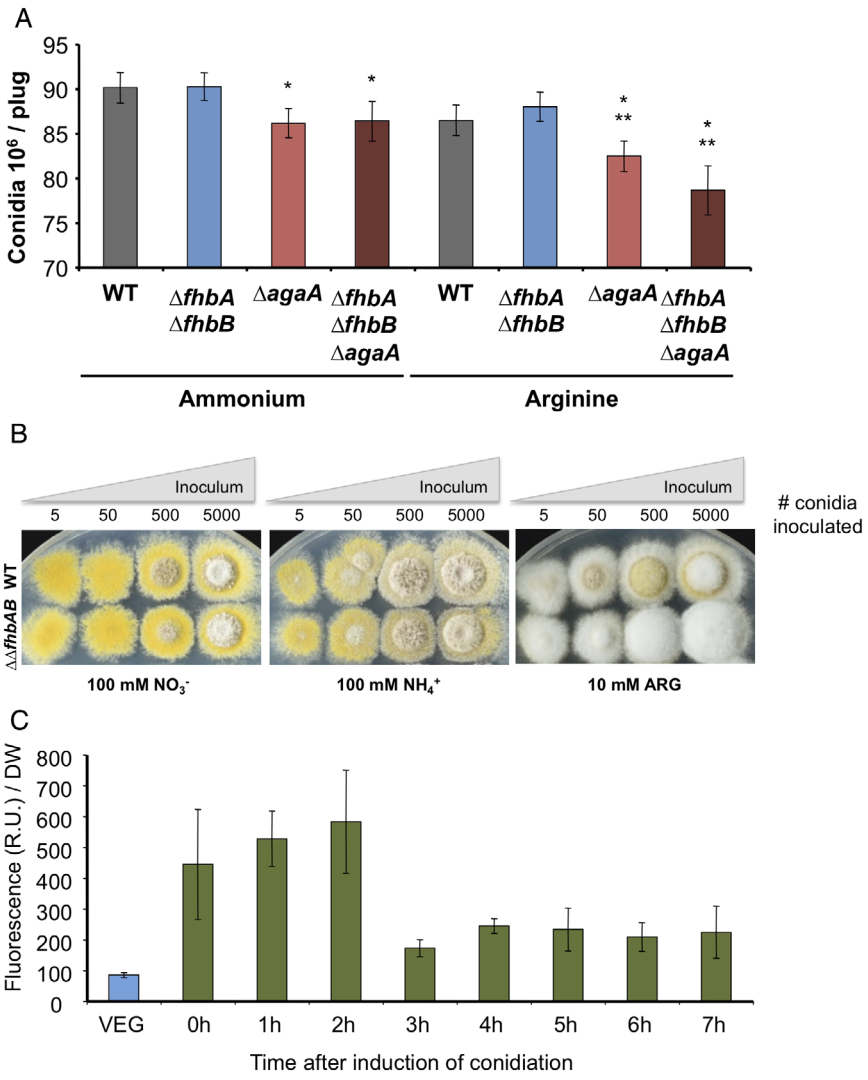

Fig. 1. Role of arginine in conidiation.
A. *A. nidulans* wild-type (WT), Δ*fhbA* Δ*fhbB*, Δ*agaA* or Δ*fhbA* Δ*fhbB* Δ*agaA* were grown in media containing ammonium or arginine as sole nitrogen source. Conidia were counted after 3 days of growth. The plot shows the average and standard error of the mean of conidial number values in at least four independent experiments. Differences with the wild-type strain grown on ammonium indicated with single asterisk and between the same strains grown in ammonium or arginine indicated with a double asterisk are statistically significant according to the Student's *t*-test and a *p*-value <0.05.
B. Serial dilutions of spore solutions of *A. nidulans* wild-type (WT) and Δ*fhbA* Δ*fhbB* strains were inoculated onto plates containing solid media supplemented with 100 mM nitrate, 100 mM ammonium or 10 mM arginine. Plates were incubated at 37°C and photographed after 3 days. Note that 10 mM arginine corresponds to 40 mN of nitrogen, while nitrate and ammonium provided 100 mN of nitrogen.
C. The wild-type strain was grown in ammonium liquid medium for 18 h to allow for competence and then transferred to ammonium solid medium to induce development. Samples of mycelial plugs were taken immediately and each hour after the induction, soaked in DAF-FM DA solution for 1 h and quantified by fluorescence. As a control samples from the vegetative culture in liquid media were used. Data were normalized against the dry weight of the mycelial plugs. Data presented are the mean and SEM (error bars) from three independent experiments.

sources. However, *dewB-mrfp* expression was delayed by about 2–3 h and only reached about half the level in media containing 10 mM arginine compared to colonies growing on ammonium or nitrate. Taken together, these results suggest that the reduction in conidiation is a specific effect of arginine supplementation.

Induction of conidiation produces an immediate NO burst

To investigate the kinetics of NO production after initialization of conidiation, *A. nidulans* was grown in liquid ammonium media for 18 h and then transferred to solid media to induce conidiation. NO was quantified following a previously reported assay (Marcos *et al*., 2016). As it can be seen in Fig. 1C, there was a significant burst of NO production upon transfer to solid media. This burst was detected immediately after the transfer to solid media and lasted for 2 h. Then, NO levels were reduced but always maintained higher than during vegetative growth in liquid media, as previously reported (Marcos *et al*., 2016). Since the fungal mycelia were transferred to ammonium media, we assumed that no nitrate was present in the media at these short times, and consequently, it suggests that the substrate for NO synthesis must be a different molecule.

Addition of L-arginine but not any of its analogues transiently increases NO synthesis

The data above suggest that other molecules in addition to nitrate must be a substrate for NO synthesis and that there is a correlation between arginine and NO synthesis during the induction of conidiation. Although no classical mammalian-type NOS that uses L-arginine as a substrate for NO production (Wu and Brosnan, 1992; Hattori

et al., 1994; Nussler *et al*., 1994) has been identified in fungi so far, there may still be an analogous system present. This speculation is also supported on our previous finding that an induction of developmental programs in *A. nidulans* is associated with an increase in the intracellular arginine pool (Marcos *et al*., 2020). This correlation could be direct, i.e. arginine would be a substrate for NO production as occurs in mammals, or indirect, and it could be restricted to the developmental programs or also functional during vegetative growth. To test these possibilities *A. nidulans* was grown in liquid media containing ammonium as sole nitrogen source for 16 h and then, NO production was monitored by addition of the NO-sensitive fluorescent probe DAF-FM for 60 min. At this stage, L-arginine and a full repertoire of its analogues were added individually to the fungal samples under test, and monitoring of the NO production continued. Addition of L-arginine resulted in a sudden and drastic increase of NO. The NO burst lasted ca. 5 min, and then it reached a plateau. This very rapid but transient increase of the NO levels upon addition of L-arginine suggests that – apart from nitrate via NR – L-arginine could also be a substrate for the synthesis of NO (Fig. 2A). Addition of at least 1 mM of arginine to the medium was required to trigger this response, which was further augmented if higher arginine concentrations were supplied (Fig. 2B). Arginine analogues did not produce this effect (Fig. 2A). Nω-Nitroarginine methyl ester (NAME) is a nitrated derivative of arginine used as an inhibitor of NOS in many studies (Rees *et al*., 1990; Alderton *et al*., 2001). Both stereoisomers of NAME (L- and D-NAME) were included in the experiment. Neither of the analogues (L-NAME, D-NAME or D-arginine) produced any effect on the NO synthesis (Fig. 2A).

The next question was whether similar effects can be obtained by feeding this fungal system with other amino acids involved in the biosynthetic pathway of arginine. Glutamate is the first compound in the biosynthetic route of arginine (Davis, 1986). The relative levels of glutamine and glutamate are determined by the N nutritional status of the cell, glutamine being a repressing nitrogen source (Legerton and Weiss, 1984; Berger *et al*., 2008). Aspartate enters directly into the urea cycle and reacts with citrulline to give argininosuccinate (see Fig. 2E for a scheme of the pathway). Proline is synthesized from ornithine after the catabolism of arginine (Davis, 1986), while lysine and methionine are not part of the arginine metabolic pathway, and can be considered as control amino acids in this experiment. Our results show that, in addition to arginine, also aspartate and glutamate produced the boost of NO, while none of the other tested amino acids resulted in a similar effect (Fig. 2C).

In mammals, an 'arginine–citrulline cycle' is operative, which is responsible for the NO burst in macrophages. The reaction of arginine with O_2 produces NO and citrulline, which is converted subsequently to argininosuccinate, when a sufficient amount of aspartate is available, and then further transformed into arginine to close the cycle (Wu and Brosnan, 1992; Hattori *et al*., 1994; Nussler *et al*., 1994; Hallemeesch *et al*., 2002) (Fig. 2E). If a similar cycle may be operative in *A. nidulans*, then not only the addition of arginine but also of citrulline should boost NO production. To test this, the cells were grown on ammonium and continuously incubated in the presence of aspartate to provide a sufficient source of this amino acid. Finally, excess citrulline was added and NO was quantified. In these experiments we found that the addition of citrulline during the NO measurement provoked a sudden boost of NO similar to what was observed with arginine (Fig. 2D). This finding would hint towards the presence of a shunt in the urea cycle analogous to the mammalian NOS involving NO generation from arginine via citrulline formation.

Role of the urea cycle genes in the NO levels

To better characterize the genetic background of this observation, we studied the role of the urea cycle genes (Fig. 2E and S2). The first biosynthetic step in the urea cycle is carried out by the ornithine carbamoyltransferase encoded by the *argB* gene in *A. nidulans* (Cybis *et al*., 1972) (Fig. 2E). The next two biosynthetic steps in the urea cycle are catalysed by ArgC (AN1883, argininosuccinate synthase) and ArgA (AN2914, argininosuccinate lyase) (Cybis *et al*., 1972). The last step in the urea cycle is the catabolism of arginine to ornithine and urea by the arginase AgaA (AN2901) (Borsuk *et al*., 1999). First, we selected a time point of 2 h after induction of the reproductive programs because at this point the levels of NO are maximal (see Fig. 1D). The biosynthetic genes *argA* and *argC* increased their expression after 2 h (2.5- and 3.2-fold respectively), while the catabolic gene *agaA* decreased the mRNA levels fourfold (Fig. S3). Although the differences are modest and not statistically significant, the trend was consistent with the higher intracellular pool of arginine and also with the burst of NO, both found after the induction of the developmental programs.

We further explored the connection between the synthesis of arginine and NO production employing mutants in the urea cycle (Fig. S2A and B). The phenotype of the deletion mutant Δ*agaA* resembled a previously isolated mutant *agaA*90 (Borsuk *et al*., 1999), i.e. it grew well on ammonium and L-ornithine as sole nitrogen sources and it showed reduced growth on L-arginine and L-citrulline. The *argB2* mutant could grow on L-citrulline and L-arginine but not on ammonium or L-ornithine. And the

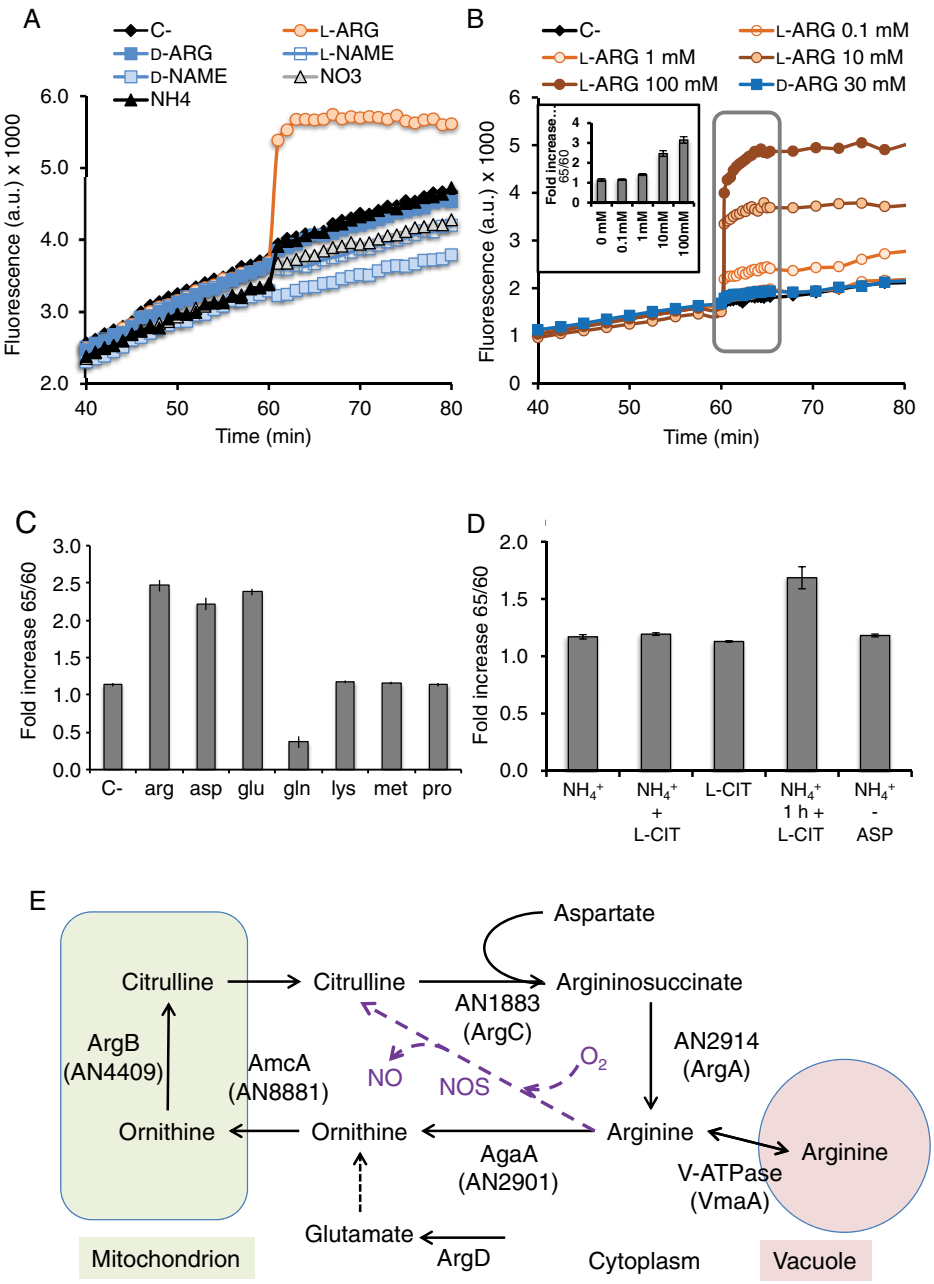

Fig. 2. NO synthesis boosts upon addition of L-arginine. The wild-type strain was grown in ammonium liquid medium. NO synthesis monitoring started with the addition of DAF-FM DA to the cultures.

A. After 1 h L-arginine or any of the indicated derivatives or nitrate or ammonium were added at 10 mM and quantification of NO continued.

B. After 1 h different concentrations of arginine (0.1–100 mM) were added to the cultures and quantification of NO continued. The negative controls consisted of the same volume of water. As additional controls 30 mM D-arginine was added as indicated. The inset graph depicts the fold increase in fluorescence between 60 and 65 min marked with a grey rectangle in the main graph, i.e. before and 5 min after the addition of the solute.

C. In this experiment, the indicated amino acids were added 1 h after the quantification of NO started and then monitoring of NO continued. The graph depicts the plot of the fold increase in fluorescence obtained 5 min after the addition. The mean and SEM (error bars) from three independent experiments is shown.

D. The wild-type strain was grown in liquid medium containing aspartate in the presence or in the absence of ammonium and/or citrulline as indicated. NO synthesis monitoring started with the addition of DAF-FM DA to the cultures. After 1 h citrulline was added to one of the ammonium + aspartate samples and quantification of NO continued. The graph depicts the plot of the fold increase in fluorescence obtained 5 min after the addition. The mean and SEM (error bars) from three independent experiments is shown.

E. The urea cycle is depicted in the diagram showing the *A. nidulans* genes and the compartmentalisation of the biosynthetic steps. The dotted line in purple colour indicates the possible NOS-like activities investigated in this work.

argC3 and *argA1* mutants grew only when L-arginine was present in the media. D-arginine could not be used as sole nitrogen source (Fig. S2B). *argD11* is an uncharacterized mutant in a step in the synthesis of glutamate (Cybis *et al*., 1972) and it did not appear to be auxotrophic for arginine [Fig. S2A and B and reference Cybis *et al*., 1972]. The wild-type strain and all the mutants in the urea cycle genes were grown on solid media containing arginine as sole nitrogen source. No significant differences in the production of NO were found between the wild type and any of the mutants (Fig. S2C). Similar results were observed by Zhang *et al*. in *Magnaporthe* (Zhang *et al*., 2015). This finding suggests that none of the urea cycle enzymes themselves is involved in the observed NO production from arginine, citrulline, aspartate or glutamate. However, block of arginine catabolism by deletion of *agaA* should lead to higher intracellular arginine levels possibly also translating into a stronger NO production, if the NO-generating system is not already saturated by the external addition of excess arginine. Consistent with this idea, the *argB2* mutant still grew after transfer from arginine-supplemented media onto arginine-free media and only revealed the auxotrophic phenotype after sporulation and re-inoculation on non-supplemented medium (Fig. 3A). This implies that a highly stable intracellular arginine pool must exist, which may partially obscure a strong effect of the *agaA* deletion mutant on NO production. However, a twofold to threefold increased NO level was indeed detected in the Δ*agaA* strain after transfer to arginine-free medium (Fig. 3B). But unexpectedly, also the *argB2* mutants showed higher levels of NO production than the wild-type strain at later time points. This was surprising, since the *argB2* mutation prevents the internal synthesis of arginine. One could speculate that in consequence to a defective internal arginine biosynthesis, this strain may de-regulate arginine storage and mobilization from the vacuoles. It is known from other fungi (Weiss, 1973, 1976; Drainas and Weiss, 1982; Davis, 1986) that vacuoles act as storage for arginine and over 90% of the arginine pools are located in the fungal vacuoles (Weiss, 1973). Indeed, when cultures of these strains were transferred from ammonium + arginine to sole arginine medium, the levels of NO were also higher in both mutants (Δ*agaA* and *argB2*) than in the wild-type strain (Fig. S4A). We also tested if arginine was capable of provoking the sudden burst of NO in both mutants. In this case, all the strains were also grown in ammonium + arginine to allow the growth of the *argB2* mutant. When L-arginine was added to the cultures during the NO measurement as previously described in Fig. 1, a burst in NO synthesis in *argB2* and Δ*agaA* mutants could also be observed. However, there were no significant differences in the fold increase between the wild-type and either mutant strain (Fig. S4B), which, together with data in Fig. S2C, shows that transfer to arginine-free media is essential for this type of assays. The data above point to a possible role of the vacuoles in the regulation of NO biosynthesis by storage of arginine. In order to gain a first insight into this possible mechanism we used a chemical genetics approach and inhibited vacuolar transport with bafilomycin, DCCD or a combination of both. Bafilomycin A1 is a specific and potent inhibitor of vacuolar H^+-ATPases (Bowman and Bowman, 2002). DCCD binds covalently and inhibits F_1F_0-ATPase, including V-ATPases (Solioz, 1984; Yang *et al*., 1999). Addition of either inhibitor alone already provoked a significant reduction in NO synthesis (Fig. 3C) and a combination of both inhibitors did not further reduce NO synthesis. These data suggest that vacuolar mobilization is involved in the NO biosynthesis route, however, if this is a direct consequence of the release of arginine or other compounds has not been tested further.

To test a putative relation between the NR pathway and the arginine-mediated NO synthesis, double mutants in *niaD*, and *argB2* or Δ*agaA* were constructed. The strains were grown on ammonium + arginine or sole arginine to allow the growth of the *argB2* strains (Fig. 3D). No major differences in NO synthesis were again observed when the different strains were grown in arginine as sole nitrogen source with the exception of the Δ*niaD* Δ*agaA* strain. However, all the mutant strains showed increased NO production compared to the wild-type strain in ammonium + arginine media, including the Δ*niaD* single mutant. The double mutation of *niaD* and *argB* did not show an additive behaviour, and the production rate was comparable to that of the single mutants.

Arginine metabolism and the synthesis of NO

To get further information on the fate of arginine after being added to the cultures, amino acid profiling was performed before and after the addition of arginine to cultures of the wild-type strain under the same conditions of the NO assays in Fig. 2. Five minutes after the addition of arginine, the levels of citrulline appeared to increase significantly, while the levels of ornithine and arginine did not change (Fig. 4). Of all the other amino acids, only the levels of glutamine increased significantly. Concomitantly, the levels of glutamate decreased, which suggests that variation in these two amino acids was a response to the availability of fresh nitrogen source in the media (Fig. 4), which is in agreement with published data (Berger *et al*., 2008). One hour after the addition of arginine, not only citrulline but also ornithine increased their levels. This increase was also observed in the case of the complete pool of amino acids and could be a general response to the increased availability of nitrogen in the media (Fig. 4; Table S1).

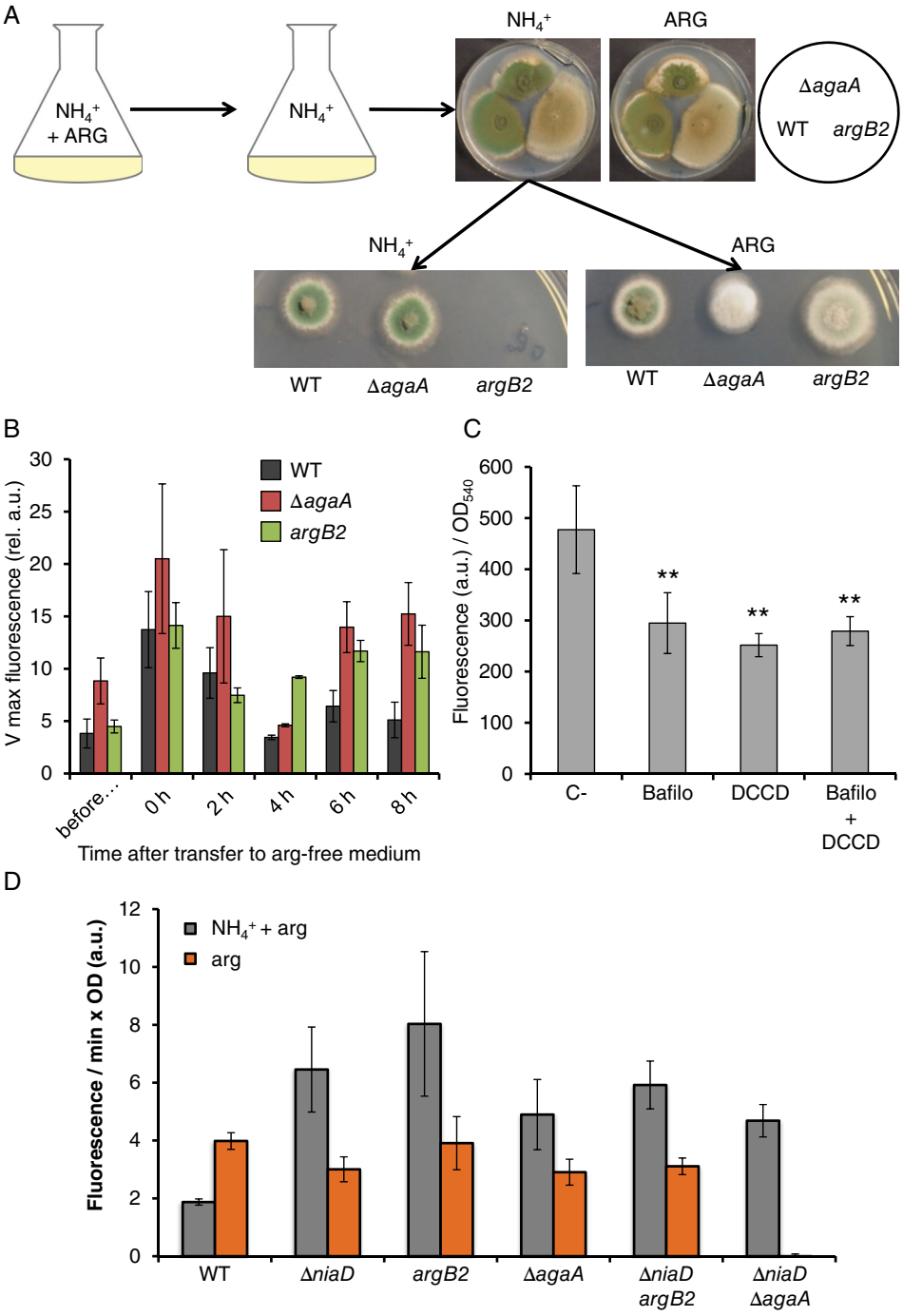

Fig. 3. Urea cycle, vacuoles and NO synthesis.
A. The *A. nidulans* wild-type, *argB2* and Δ*agaA* strains were grown in liquid media containing ammonium + arginine to allow the growth of *argB2*, and then, transferred to liquid media containing only ammonium as sole nitrogen source. After 8 h mycelia from all three ammonium liquid cultures were transferred to plates containing ammonium or arginine as sole nitrogen sources. Conidia from the colonies were picked with toothpicks and then inoculated into fresh new plates containing ammonium or arginine as sole nitrogen sources.
B. After the transfer of the three strains to sole ammonium liquid media, NO production was monitored every 2 h for 1 h by addition of DAF-FM to the cultures.
C. The wild-type strain was grown in ammonium liquid minimal medium. Bafilomycin, DCCD or both were added to the cultures and after 1 h, monitoring of the NO production started by addition of DAF-FM DA.
D. The indicated strains were grown in arginine or ammonium + arginine liquid media and NO production was monitored by addition of DAF-FM for 1 h. Data presented are the mean and SEM (error bars) from three independent experiments.

The fast and specific appearance of citrulline in the intracellular amino acid pools would be consistent with a NOS-like activity in *A. nidulans*. Indeed, the conversion of arginine into citrulline has been employed in numerous publications as a read-out of NOS activity in cell-free extracts. There are NADPH-P450 reductases in fungi (de Vries *et al*., 2017), but numerous attempts to identify NOS-like sequences in fungal species have so far failed to reveal a clear homologue [(Samalova *et al*., 2013; Pengkit *et al*., 2016) and our own unpublished work]. As genetic evidence is missing, we set out and tried to recapitulate the cellular NO production in cell-free extracts as a first step towards a possible proteomics-based enzyme discovery. We thus analysed protein extracts of the wild-type strain and tested NO synthesis biochemically recreating the conditions for NOS activity employed in assays using mammalian extracts (Fig. 5). In a first step, we added the fluorescent NO-specific dye DAF-FM to the

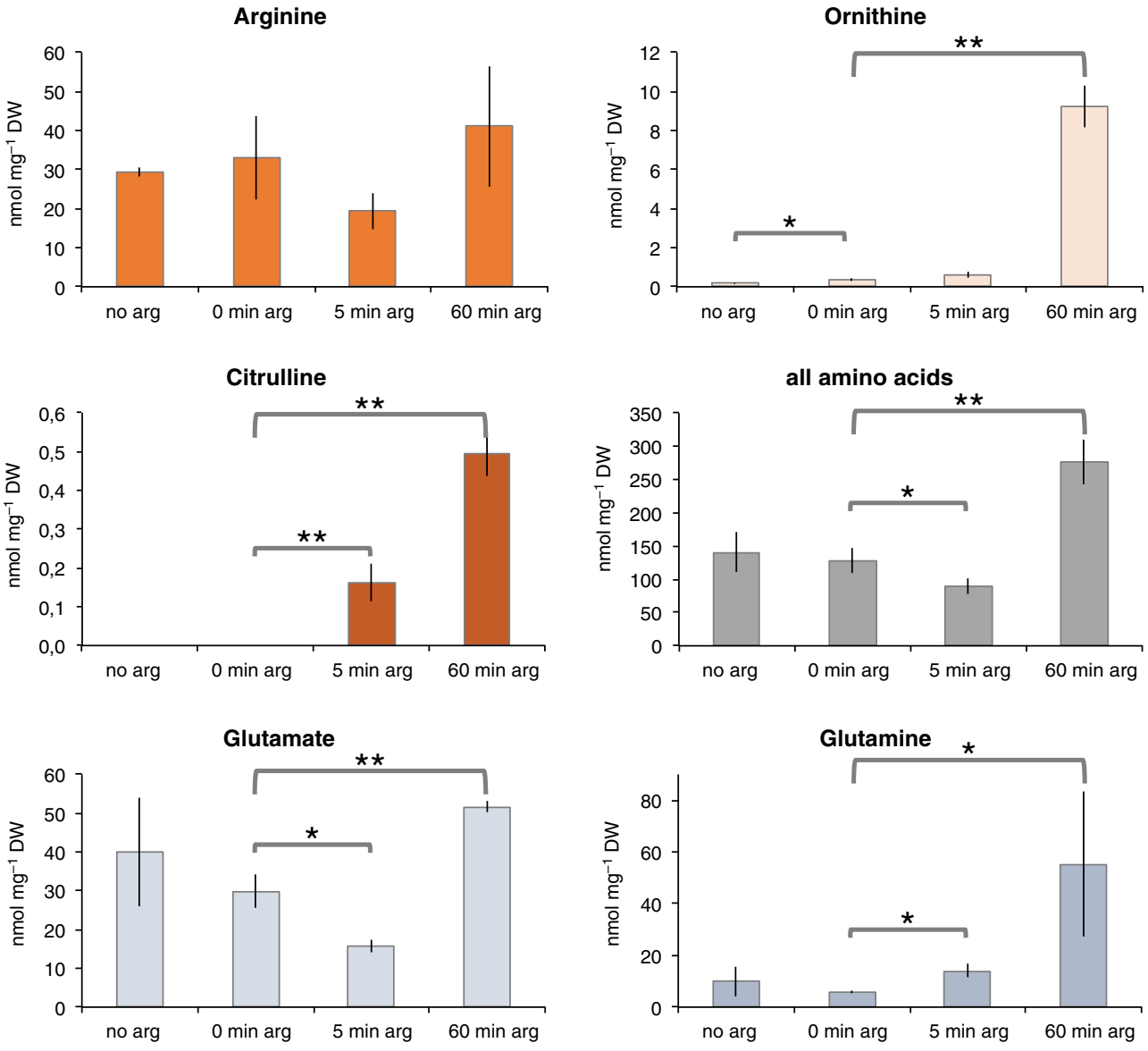

Fig. 4. Amino acids profiling after addition of arginine. *Aspergillus nidulans* was grown vegetatively in liquid minimal media containing ammonium as sole carbon source. Fungal mycelia were harvested before, and immediately (0 min), 5 min and 60 min after the addition of arginine. Amino acids were extracted from the cells and quantified by HPLC. Data presented are the mean and SEM (error bars) from three to four independent experiments. Table S1 shows the concentration of all amino acids.

crude protein extracts and this revealed a slight increase in fluorescence over the background when arginine was added to the assay. But strikingly a very strong fluorescence was detected when the biochemical assay was amended with Ca^{2+}, calmodulin and tetrahydrobiopterin (THB), which are cofactors known to be required for mammalian NOS activity. In this case a significant increase in NO production was observed (Fig. 5A). The production of NO is likely to be derived from enzymatic activity as it did not occur when the protein extracts were heated for 10 min at 95°C (Figs. 5A and see S5A for a more detailed graph). To study the dependence of the NO production on arginine, the extracts were first dialyzed to remove free arginine and then the assay was carried out with or without arginine supplementation. The results shown in Fig. 5B revealed that the sole presence of THB was sufficient to elicit a strong induction of NO synthesis, additional arginine supplementation did not further increase the signal. Also in this case, heat-inactivated extracts had no activity. The addition of arginine to the reaction mixture containing THB also elicited a strong induction of NO synthesis that was comparable to THB alone, but displaying a higher statistical significance compared to the control (the reaction mixture

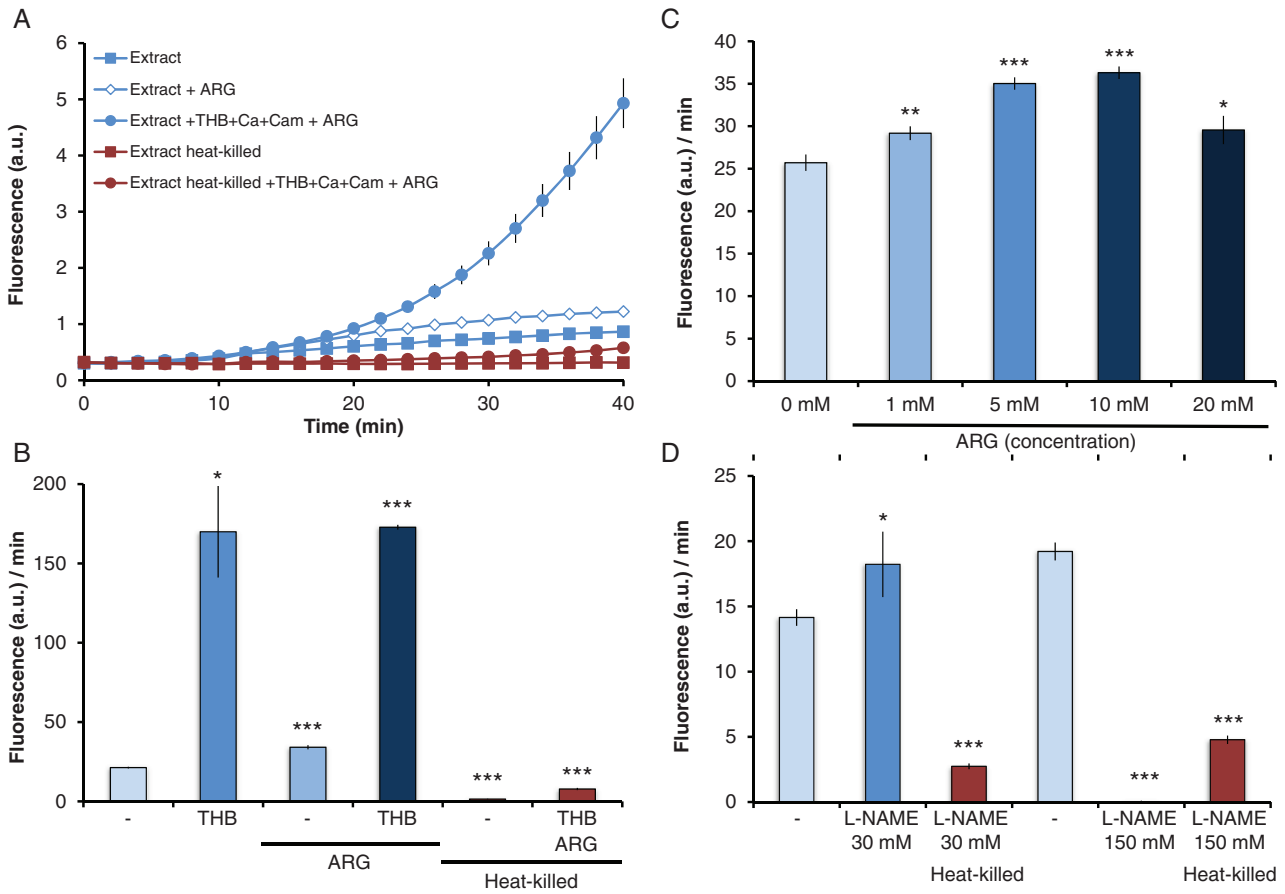

Fig. 5. In vitro NO assays. Protein extracts were obtained from vegetative cultures of *A. nidulans* grown in ammonium liquid media. Protein extracts were pre-incubated with the NO-sensitive probe DAF-FM DA and the reaction was started by addition of 10 mM arginine or phosphate buffer ('-', as negative control) to the reaction mixture. Co-factors (namely, calmodulin, calcium and/or TBH) were included in the reaction mixture from the beginning, when indicated. Negative controls were obtained by heat inactivation of the protein extracts at 95°C for 10 min ('heat-killed').
A. Protein extracts were pre-mixed with the cofactor, and the reaction was started by addition of NADPH and DAF-FM. Arginine was added 10 min after the reaction started.
B. Proteins extracts were pre-mixed with or without THB. The graph depicts the increase in the DAF-FM fluorescence per minute after the addition of arginine to the reaction mixture at 10 min.
C. The reaction mixture contained only protein extracts. The graph depicts the increase in the DAF-FM fluorescence per minute after the addition of the different concentrations of arginine to the reaction mixture at 10 min.
D. Protein extracts were pre-mixed with buffer or 30 mM or 150 mM of the NOS inhibitor L-NAME. After 10 min the reaction was started by the addition of DAF-FM and NADPH. The graph depicts the increase in the DAF-FM fluorescence per minute. Negative controls were obtained by heat inactivation of the protein extracts at 95°C for 10 min ('heat-killed'). Data presented are the mean and the SEM (error bars) from four independent biological samples (*t*-test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

containing only the protein extract), suggesting that addition of arginine together with THB elicited more reproducible NO synthesis. The fact that the protein extracts were capable of producing NO in the absence of externally added arginine or any other substrate suggests that the substrate was still present in the extracts, although the samples were dialyzed. Actually, a comparison between the NO production in dialyzed versus non-dialyzed samples revealed that the non-dialyzed samples produced more NO than the dialyzed ones (Fig. S5A). Since dialyzed samples are still capable of producing NO, it is feasible to assume that the dialysis process eliminated only a fraction but not all of the arginine present in the protein extracts. Indeed, addition of 5 and 10 mM arginine to dialyzed protein extracts resulted in the same levels of NO production than addition of 1 and 5 mM to non-dialyzed ones respectively (Fig. S5B). In the absence of externally added THB, there was a direct correlation between the concentration of arginine added to the reaction mixture and the level of NO synthesis up to 10 mM arginine (Fig. 5C). The data above point to arginine as the substrate for NO synthesis. To confirm this, we employed the mammalian NOS inhibitor L-NAME, which is an analogue of arginine (specifically Nω-Nitro-arginine methyl ester). 30 mM of L-NAME did not inhibit the NO synthesis reaction (Fig. 5D). We reasoned that being an arginine

analogue, it could be acting as a competitive inhibitor, and that due to the arginine levels in the fungal cells and in the cell extracts, higher concentrations of L-NAME may be required to inhibit NO synthesis. Indeed a concentration of 150 mM of L-NAME completely inhibited NO synthesis in this assay (Fig. 5D). Altogether these results strongly suggest that a NOS-like THB/Ca^{2+}/CaM dependent NO generation pathway using arginine as a substrate exists in *A. nidulans*.

Polyamines induce the synthesis of NO

The urea cycle also provides intermediates for the synthesis of other NO-generating substrates. Specifically, synthesis of NO from polyamines has been reported in plants, although the pathway is not characterized in detail (Tun *et al*., 2006; Wimalasekera *et al*., 2015; Recalde *et al*., 2021). The biosynthetic pathway for polyamines starts from ornithine and/or arginine (Tabor and Tabor, 1985; Rocha and Wilson, 2019; Recalde *et al*., 2021), and consequently it is connected to the urea cycle (Fig. A). Several attempts were performed to study the synthesis of NO from polyamines using *A. nidulans* fungal cells with little success, probably due to the poor transport of spermidine and spermine in *A. nidulans* (Spathas *et al*., 1982; Spathas *et al*., 1983). Therefore, we obtained protein extracts of *A. nidulans* vegetative cells and employed an *in vitro* assay for the synthesis of NO, which did not require active transport into the cell (Fig. 6B). Addition of any of the polyamines provoked an increase in the NO synthesis using the *in vitro* assay. The best polyamine boosting NO synthesis was putrescine followed by spermidine. In order to try to identify which one of the polyamines is employed as a substrate for the synthesis of NO, we took advantage of the polyamine auxotrophic mutants affected in *puA* (ornithine decarboxylase) (Sneath, 1955) or *spdA* (spermidine synthase) (Jin *et al*., 2002) (Fig. S6A). The NO synthesis assays using protein extracts of the wild-type strain and the *puA2* and Δ*spdA* mutants revealed that NO synthesis is generally reduced in these mutants. However, these assays could not pinpoint which of the polyamines were employed as substrate for NO synthesis (Fig. S6B).

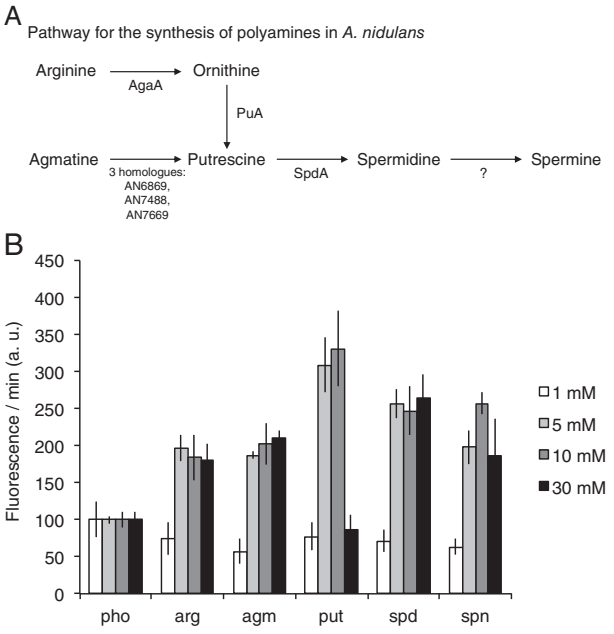

Fig. 6. Induction of NO synthesis by polyamines.
A. The pathway for the synthesis of polyamines is depicted in the diagram showing the characterized *A. nidulans* genes and predicted homologues.
B. Protein extracts were obtained from 16 h vegetative cultures of *A. nidulans*. Extracts were pre-incubated with the NO-sensitive probe DAF-FM DA and the reaction was started by addition of buffer (Pho, as negative control), arginine (Arg, positive control) and the indicated concentrations of the polyamines to the reaction mixture (Agm, agmatine; Put, putrescine; Spd, spermidine; Spn, spermine). Data presented are the mean and the SEM (error bars) of 4–6 independent experiments.

Discussion

Previous works by others and in our own lab suggest that NO signalling regulates developmental processes (Samalova *et al*., 2013; Marcos *et al*., 2016; Pengkit *et al*., 2016; Ding *et al*., 2020). Here we found a correlation between the NO levels and presence of arginine in the medium that may indicate the presence of an animal-like NOS generating system in fungi. Using conidiation as biological reporter system we first found that conidiation levels differ between the N-sources ammonium, nitrate and arginine. Noticeably, very high concentrations of arginine prevented conidiation (but not sexual reproduction), which did not happen employing nitrate or ammonium as nitrogen sources. In the same experimental context NO levels were found to boost upon transfer of mycelia from submerged liquid culture to a solid surface to induce the reproductive programs, NO levels are reduced on ammonium, but not on arginine at the onset of conidiation. Moreover, high concentrations of arginine reduced the conidiation levels, an effect that was more pronounced in a flavohemoglobin mutant.

Previous works demonstrated that NR contributes to NO synthesis (Marcos *et al*., 2016; Ding *et al*., 2020), and that the levels are regulated by light (Marcos *et al*., 2020). This work provides evidence of the existence of one additional pathway generating NO during the developmental program, and that this pathway is fuelled by arginine. Indeed, in this work we found that when arginine was added directly to the NO assay, a burst of NO could be observed. The effect was very rapid and transient. Note that the fold increase in NO production obtained experimentally is a combination of transport

of arginine into the cells, and disappearing of arginine by synthesis of NO, catabolism by arginase and transport into the vacuoles. The NO burst was also obtained by addition of some of the intermediates in its biosynthetic pathway tested (i.e. glutamate, citrulline and aspartate) but not of other amino acids, such as methionine, proline or lysine, or with D-arginine. Citrulline feeds directly into the urea cycle, reacting with aspartate to give argininosuccinate, the immediate precursor of arginine. Both citrulline and aspartate provoked a NO burst that is smaller than arginine. This is analogous to the findings in mammals, where an 'arginine–citrulline cycle' (also called 'citrulline–NO cycle') was postulated and proven (Hallemeesch *et al*., 2002). In mammals, this cycle can operate only when sufficient aspartate is available to keep the urea cycle running, which requires an active TCA cycle. The arginine-citrulline shunt plays an important role in maintaining the intracellular concentrations of arginine when prolonged NO production is necessary, for example when iNOS is expressed in macrophages (Wu and Brosnan, 1992; Hattori *et al*., 1994; Nussler *et al*., 1994). The connection between this arginine-citrulline shunt and the synthesis of NO is further supported by the transient and functional association of argininosuccinate synthase and argininosuccinate lyase with NOS (Solomonson *et al*., 2003; Erez *et al*., 2011).

Our current work in *A. nidulans* using amino acid profiling suggests that upon addition, arginine could be directly metabolized to citrulline, which is concordant with the existence of an arginine-NO pathway. *In vitro* assays using cell extracts are also concordant with the existence of an arginine-NO pathway. Furthermore, addition of THB, which is required for mammalian NOS activity *in vitro*, further stimulated the production of NO in *Aspergillus* cell extracts, and the mammalian NOS inhibitor L-NAME could inhibit NO synthesis *in vitro*. Taken all together these data strongly suggest the existence of a fungal arginine-NO pathway, analogous to the mammalian one. However, published works in *M. oryzae* and *N. crassa* (Samalova *et al*., 2013; Pengkit *et al*., 2016) and unpublished work from our lab in *A. nidulans* could not so far identify candidate genes, which encode enzymes with analogous functions to the mammalian NOS genes. Similarly, in plants NOS-like sequences have not been clearly identified (Jeandroz *et al*., 2016).

Availability of arginine is a key factor in NO synthesis in mammals and plants (Flores *et al*., 2008; Caldwell *et al*., 2018). Similarly, in *Aspergillus* deletion of *agaA* increased NO levels, and the NO burst early during the transition from vegetative growth to development was associated to slight changes in transcription of arginine metabolic (induced) and catabolic (repressed) genes, which is in agreement with data on other organisms (Wu and Morris Jr., 1998). In fungi, over 90% of the arginine pools are stored in the vacuoles, which control the cellular demands for arginine (Weiss, 1973, 1976; Drainas and Weiss, 1982). Addition of ATPase inhibitors blocking vacuolar transport reduced the synthesis of NO. Although the effects of these inhibitors can be pleiotropic, it provides some preliminary evidence that the vacuolar transport of arginine could be in part responsible for controlling the synthesis of NO by this route. The vacuolar ATPase was first identified in *N. crassa* by RIP mutation (Bowman *et al*., 2000) and later on in *A. nidulans* (Melin *et al*., 2004). In both organisms, mutation of the V-ATPAse results in severe growth and morphological defects accompanied by absence of reproductive structures. The storage of arginine in the vacuoles could also explain the unexpected growth of *argB2* strain on plates containing ammonium as sole nitrogen source, after transfer from liquid medium, assuming that mobilization of arginine from the vacuoles may happen preferentially in certain stages of the life cycle, which would also be compatible with the NO burst during the transition from vegetative growth to conidiation.

We also found in this work that polyamines increased NO synthesis. The role of polyamines in fungal biology is not clear yet, and for example, there are gaps in the knowledge about the biological roles of putrescine (Rocha and Wilson, 2019), despite mutants blocked in putrescine synthesis can grow in the presence of spermidine (Fig. S6A). We found that all polyamines increased the synthesis of NO. Similar results were found in *Arabidopsis* (Tun *et al*., 2006), although the role of the polyamines in NO is also not clear yet (Recalde *et al*., 2021). Mutation of one of the known genes in the polyamine biosynthetic genes, *puA* or *spdA*, resulted in a general but slight decrease of NO production, which did not allow us to discern which one of the polyamines could be a substrate for NO synthesis. Alternatively, there could be more than one substrate in this pathway. For example, amine oxidases are responsible for the oxidative deamination of both spermidine and spermine, and they are potential enzymes responsible for the synthesis of NO from polyamines (Wimalasekera *et al*., 2015; Murray Stewart *et al*., 2018), although another work found that a copper amine oxidase contributed to NO synthesis by affecting arginine availability (Groß *et al*., 2017). The *puA2* mutant required agmatine, putrescine or spermidine for growth, and it could not grow with spermine. The Δ*spdA* mutant could only grow when spermidine, but not any other polyamine, was present in the media (Fig. S6A). It has been reported that spermine can be converted back to spermidine and spermidine back to putrescine in a two-steps process (Murray Stewart *et al*., 2018). However, our observations suggested that either the polyamine biosynthetic pathway was not reversible, or that it was not efficient enough to support

A. nidulans growth, as the *puA2* and the Δ*spdA* mutants could not grow in the presence of spermine.

In conclusion, here we provide evidences that there is a functional arginine-dependent route for the synthesis of NO in the fungal kingdom, analogous to the mammalian NOS pathway, and that also polyamines, which are synthesized from urea cycle intermediates, induce the synthesis of NO.

Experimental procedures

Strains, media and culture conditions

Strains used in this study are listed in Table S2. Strains were grown in complete or minimal media containing the appropriate supplements at 37°C (Cove, 1966). One percent glucose was used as carbon source. Ammonium tartrate, sodium nitrate and/or arginine were used as nitrogen sources at 5, 10 and 3 mM respectively, unless otherwise indicated. Diaminofluorescein-FM diacetate (DAF-FM DA), bafilomycin and DCCD were purchased from Sigma. Strains DKA149, DABO65, DABO66 and DABO103 were obtained by sexual crosses following standard procedures (Pontecorvo *et al*., 1953). Strain DKA150 was constructed by transformation of a Δ*agaA::pyrG* cassette (Program Project grant GM068087, PI Jay Dunlap) obtained from the Fungal Genetics Stock Center (www.fgsc.net) in the Δ*nkuA* mutant strain A1155 (MAD1739) following standard procedures (Nayak *et al*., 2006; Alvarez-Escribano *et al*., 2019).

RNA isolation and real-time RT-PCR

Isolation of RNA and quantification of mRNA were performed as previously described (Ruger-Herreros *et al*., 2011) with some modifications. *Aspergillus* mycelia (100–200 mg) were disrupted in 1 ml of TRI reagent (Sigma) with 1.5 g of zirconium beads using a cell homogenizer (FastPrep-24, MP Biomedicals). Cell debris was removed by centrifugation. Supernatants were extracted with chloroform and RNA was precipitated with isopropanol. RNA samples were treated with RNAse-free DNAse and checked for the absence of DNA by qPCR. cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad). The primers employed for real-time qPCR are detailed in Table S3. Real-time qPCR experiments were performed using the SYBRgreen kit (BioRad). The fluorescent signal obtained for each gene was normalized to the corresponding fluorescent signal obtained with the actin gene. Expression data are the average of at least three independent replicates.

Physiological experiments

Strains were grown in liquid medium for 18 h at 37°C and then transferred to solid media. Plates were incubated at 37°C under light for conidiation conditions, or alternatively, they were sealed and incubated in the dark for sexual developmental conditions. Plugs were cut out from the plate 72 h after induction of development. Conidia in the plugs were resuspended in Tween 0.1% buffer and counted. Data shown are the average of at least four independent experiments.

Amino acid profiling

Amino acid analysis was performed as previously described (Berger *et al*., 2008; Marcos *et al*., 2020). Briefly, amino acids were extracted with boiling 75% ethanol three times and the supernatants were pooled after spinning down the cell debris(Berger *et al*., 2008). Amino acids were analysed by HPLC after derivatization with *6-aminoquinolyl*-N-hydroxysuccinimidyl carbamate in a chromatographer equipped with a pre-column WAT044380, Waters AccQ-Tag column 3.9 × 150mm WAT052885, an automatic injector (Waters Alliance 2695), and a scanning fluorescence detector (Waters 474). Five dilutions (7.8, 15.61, 31.25, 62.5 and 100 mM) of an equimolar solution of amino acids in HCl (Amino Acid Standard H, Pierce) were used as standards. Integration and processing of data were performed with the Waters Millennium 32 software.

NO quantification and plate reader experiments

Plate reader experiments were performed as previously described (Marcos *et al*., 2016; Canovas *et al*., 2017). NO was quantified in samples by using the NO-sensitive fluorescent dye DAF-FM diacetate (Invitrogen) following the manufacturer's instructions as previously reported (Marcos *et al*., 2016). The plate reader employed in this work a Synergy HT Multi-mode Microplate Reader (Biotek) with the appropriate filter sets and data were analysed with Gen5™ Data Analysis Software. In all experiments, data were normalized to dry weight or absorbance as indicated. Experiments were repeated at least three times and performed in duplicates or triplicates, depending on the experiment.

Biochemical assays of NOS-like activity and NO synthesis from polyamines in fungal cell extracts

Mycelia were broken using a fastprep device followed by separation of the cell debris by centrifugation. Phosphate buffer 10 mM pH 7.0 was used as solvent. Phenylmethylsulfonyl fluoride leupeptin and pepstatin

were included in the buffer as protease inhibitors. Dialysis was performed in dialysis bags (12 kDa, Sigma) against phosphate buffer 10 mM pH 7.0 for 36 h at 4°C with three changes of the dialysis buffer. Crude protein extracts were quantified by BioRad Bradford, then diluted down prior to assays. A typical assay contains protein extracts (50 μg ml^{-1}) in phosphate buffer, DAF-FM DA and 150 μM NADPH. Reactions were started and arginine or polyamines added 10–15 min after, as indicated. Fluorescence was recorded in a Synergy HT microplate reader for 20–40 min at 37°C. 0.5 mM $CaCl_2$, 10 μM bovine calmodulin (Sigma-Aldrich) and 50 μM (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride (THB) (Sigma-Aldrich) were used as co-factors in the reaction mixture before the reaction started when indicated.

Statistical procedures

Data were analysed using Excel. Standard error of the mean (SEM) was calculated using at least three independent biological replicates. Paired-sample *Student's t*-test was employed to compare the statistical significance between different treatments.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supporting Information.

Fig. S1. Real-time monitorization of conidiation in different nitrogen sources. DewB is a hydrophobin that is expressed specifically during conidiation (Grunbacher *et al*., 2014). *A. nidulans dewB-mRFP* was grown in minimal media containing 10 mM ammonium, 10 mM nitrate or 3 mM arginine as sole nitrogen sources. By multiplexing absorbance as a measurement of growth and fluorescence generated by DewB-mRFP were recorded every hour for 40 h. Absorbance is indicated in black and fluorescence in red.

Fig. S2. Role of the urea cycle in the synthesis of NO. (A) Growth of mutants in the urea cycle on minimal media plates containing different nitrogen sources or intermediates in the urea cycle. (B) Growth of mutants in the urea cycle on minimal media containing different nitrogen sources or intermediates in the urea cycle was monitored every hour by measuring the absorbance at 630 nm. (C) *A. nidulans* strains were grown in arginine solid minimal media. NO was quantified with the fluorescent probe DAF-FM DA. The mean and s.e.m. (error bars) from 3–4 independent experiments is shown.

Fig. S3. Developmental regulation of the urea cycle genes. *A. nidulans* wild-type strain was grown vegetatively in ammonium liquid minimal medium for 18 h and then transferred to ammonium solid medium containing to induce conidiation or sexual development. Samples were taken at the indicated time points for ARN isolation. *argB*, *argC*, *argA* and *agaA* expression was quantified by real time RT-qPCR. Data were normalized against the expression of the actin

gene (*actA*). The plots show the average and s.e.m. of the relative expression values in at least 3 independent experiments.

Fig. S4. Urea cycle and NO synthesis. (A) The *A. nidulans* wild-type, *argB2* and Δ*agaA* strains were grown in liquid media containing ammonium + arginine to allow the growth of *argB2*, and then, transferred to liquid media containing ammonium, proline or arginine as sole nitrogen source. NO production was monitored immediately after the transfer for one hour. (B) The three strains were grown in liquid media containing ammonium + arginine to allow the growth of *argB2* and then transferred to liquid media containing ammonium as sole nitrogen source. NO production started to be monitored immediately after the transfer. After one hour of NO quantification 10 or 100 mM arginine were added to the cultures and quantification of NO continued. The graph depicts the plot of the fold increase in fluorescence obtained five minutes after the addition of arginine. Data presented are the mean and s.e.m. (error bars) from 3 independent experiments.

Fig. S5. Comparison between the synthesis of NO in dialyzed vs. non-dialyzed protein extracts. *A. nidulans* was grown in ammonium liquid medium at 37°C for 20 h. Protein extracts made as described in Materials and Methods were divided in two, half of each sample was dialyzed, while the other half was not dialyzed to do parallel comparisons. (A) Protein extracts were tested at a concentration of 10 and 50 μg protein/ml for NO production by the addition of DAF-FM. As a negative control, 50 μg/ml protein extracts were heat inactivated for 10 min at 95°C. (B) Reactions using 50 μg/ml protein extracts started by addition of DAF-FM. Arginine at the indicated concentrations was added to the reaction after 10 min and the reaction continued. The graphs depict the mean fluorescence and the s.e.m. (error bars) from 4 independent biological samples.

Fig. S6. Role of polyamine synthesis in the synthesis of NO. (A) Growth of polyamine auxotrophic mutants on minimal media plates containing different polyamines and nitrogen sources. (B) *A. nidulans* strains were grown in ammonium + spermidine liquid media for 16 h and then transferred to ammonium media. NO was quantified with the fluorescent probe DAF-FM DA and the indicated polyamines were added. The graph depicts the mean of the increase in fluorescence per min after the addition of the solutes and the s.e.m. (error bars) from 3–4 independent experiments.