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Transcription of purine transporter genes is activated during the isotropic growth phase of *Aspergillus nidulans* conidia

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Summary

Aspergillus nidulans **possesses three well-characterized purine transporters encoded by the genes *uapA*, *uapC* and *azgA*. Expression of these genes in mycelium is induced by purines and repressed by ammonium or glutamine through the action of the pathway-specific UaY regulator and the general GATA factor AreA respectively. Here, we describe the regulation of expression of purine transporters during conidiospore germination and the onset of mycelium development. In resting conidiospores, mRNA steady-state levels of purine transporter genes and purine uptake activities are undetectable or very low. Both mRNA steady-state levels and purine transport activities increase substantially during the isotropic growth phase of conidial germination. Both processes occur in the absence of purine induction and independently of the nitrogen source present in the medium. The transcriptional activator UaY is dispensable for the germination-induced expression of the three transporter genes. AreA, on the other hand, is essential for the expression of *uapA*, but not for that of *azgA* or *uapC*, during germination. Transcriptional activation of *uapA*, *uapC* and *azgA* during germination is also independent of the presence of a carbon source in the medium. This work establishes the presence of a novel system triggering purine transporter transcription during germination. Similar results have been found in studies on the expression of other transporters in *A. nidulans*, suggesting that global expression of transporters might operate as a general system for sensing solute availability.**

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Introduction

In *Aspergillus nidulans*, conidiospores are asexually produced, dormant, mononucleate cells that are characterized by very low rates of metabolism. Dormancy breaks if the environment is suitable for growth, conidiospores swell rapidly, the nucleus reorganizes, and a germ tube emerges to develop eventually into a growing hypha. Germination, the process by which dormant spores give rise to mycelia, has been divided into three phases, the isotropic growth phase (I), the stage of polarity establishment (PE) and the stage of polarity maintenance (PM) (Momany, 2002 and references therein). The isotropic growth phase is characterized by conidiospore swelling, nuclear decondensation and changes in surface properties reflected by increased adhesion. Many biochemical processes become highly active very early during (20 min) this phase, including trehalose metabolism, respiration, nucleic acid and protein synthesis, hydration and release of extracellular enzymes (Bainbridge, 1971; D'Enfert, 1997). However, very little is known about the involvement of these events in the initial signalling mechanisms controlling germination. In fact, it is difficult to differentiate between the true early signalling events and the plethora of metabolic and housekeeping activities that take place at the very beginning of the process. Any mutation affecting either of the two would lead to a defective germination phenotype (Osherov and May, 2001). A model for conidial germination is emerging, in which the first essential step is the uptake of a carbon source and the subsequent activation of the *rasA* and cAMP signalling pathways (Osherov and May, 2000; 2001; Fillinger *et al.*, 2002). This, and possibly other, pathways activate the translation machinery making use of pre-existing riboso-

mal subunits, producing a rapidly growing pool of newly synthesized proteins and metabolites. These metabolic changes are reflected in morphological changes leading to entry into the cell cycle and hyphal development.

Conidial germination seems to involve the transcriptional activation of not only housekeeping genes, such as the actin-encoding gene *acnA*, but also genes encoding proteins that may be involved in 'sensing' the presence of nutrients (Tazebay *et al*., 1997 and work therein). Two older physiological studies have shown that nitrate and ammonium transport activities in *A. nidulans* are very low in resting conidiospores, increase dramatically during germination to reach a maximum associated with germ tube emergence and drop to basic levels in mycelium (Cook and Anthony, 1978; Brownlee and Arst, 1983). In a more recent study, we have shown that PrnB, the proline transporter of *A. nidulans*, is not expressed in resting conidiospores but is transcriptionally activated during the isotropic growth phase of germination (Tazebay *et al*., 1995; 1997). *prnB* transcriptional activation during germination, although it partially responds to physiological signals such as amino acid starvation or proline induction, results from a novel control. This control is independent of the known transcription regulators PrnA and CpcA, operating clearly after the isotropic growth phase (Tazebay *et al*., 1997). Very recently, an aspartate/glutamate transporter (A. Apostolaki and C. Scazzocchio, unpublished) and a uracil transporter (S. Amillis and G. Diallinas, unpublished) have also been found to be activated during the isotropic growth phase of germination of *A. nidulans*.

In this work, we investigate further the mechanisms underlying nutrient uptake during *A. nidulans* conidial germination by studying the expression of three well-characterized purine transporters, encoded by the genes *uapA*, *uapC* and *azgA.* All three genes have been cloned, their sequence established and their transcriptional regulation in mycelia studied in detail (Darlington and Scazzocchio, 1967; Diallinas and Scazzocchio, 1989; Gorfinkiel *et al*., 1993; Diallinas *et al*., 1995; Ravagnani *et al*., 1997; Cecchetto *et al*., 2004). UapA and UapC belong to a widespread family of nucleobase transporters, known as NAT, with representatives in all kingdoms (Diallinas *et al*., 1998; De Koning and Diallinas, 2000). AzgA defines a novel protein family, with homologues of unknown function from prokaryotes, fungi and plants (Cecchetto *et al*., 2004). All three transporters have also been studied kinetically by radiolabelled purine uptake studies in germinating conidiospores and young mycelium (Diallinas *et al*., 1995; 1998; Meintanis *et al*., 2000; Cecchetto *et al*., 2004). UapA is a high-affinity, high-capacity transporter of uric acid and xanthine. UapC is a high-affinity, moderate-capacity transporter of uric acid and xanthine, but can also transport, albeit with very low capacity, all other purines. AzgA is a high-affinity, high-capacity transporter of adenine, hypoxanthine and guanine. The specific molecular determinants for the substrate specificity of UapA have been partially characterized (Diallinas *et al*., 1998; Meintanis *et al*., 2000; Amillis *et al*., 2001; M. Koukaki and G. Diallinas, unpublished).

Regulation of expression of all three purine transporter genes in developed mycelium has shown that all genes are expressed at a basal level when a non-repressing nitrogen source is present in the growth medium. In the presence of purines, which can be used as nitrogen sources, the expression of all genes is induced (Gorfinkiel *et al*., 1993; Diallinas *et al*., 1995; Cecchetto *et al*., 2004). Induction is mediated through the action of the pathway-specific regulatory protein UaY, an activator of the zinc binuclear cluster type (Scazzocchio *et al*., 1982; Diallinas and Scazzocchio, 1989; Gorfinkiel *et al*., 1993; Oestreicher and Scazzocchio, 1995; Suarez *et al*., 1995). Preferred nitrogen sources such as ammonium or glutamine repress the expression of all purine transporter genes. Repression is mediated through the inactivation of the general GATA-like transcription factor AreA necessary for the expression of a plethora of genes encoding proteins necessary for nitrogen source utilization (Arst and Cove, 1973; Kudla *et al*., 1990; Ravagnani *et al*., 1997). Ammonia and/or glutamine act by preventing AreA-mediated transcriptional activation at a number of levels including *areA* transcription, mRNA stability and binding to DNA (Wilson and Arst, 1998; Morozov *et al*., 2001).

Work presented here demonstrates the existence of a novel control system that operates independently from UaY, and for *azgA* and *uapC* also from AreA, to elicit the transcription of purine transporter genes early during conidial germination.

Results

The transcription of purine transporter genes is elicited during the isotropic phase of germination

We studied the expression of UapA, UapC and AzgA purine transporters in resting and germinating conidiospores, until the onset of germ tube emergence. Germination was carried out in minimal media (MM), supplemented with urea as the sole nitrogen source, at 37°C. To monitor germination stages, conidiospores from resting conidiospores or cultures growing for 1, 2, 3, 4, 5 or 6 h were observed under an epifluorescence microscope, directly or after nuclei staining. For each stage, 250–500 conidiospores were observed for each strain used in this analysis. Resting and 1 h germinating conidiospores were small, with condensed single nuclei and no adhesion properties. At 2 h, 13% of germlings were swollen and showed adhesion properties, but all still have a single nucleus. At 3 h, 27% of conidiospores remained

small and condensed, 69% were swollen and showed adhesion properties, and 4% showed two nuclei, of which the great majority had a visible germ tube emerging. At 4 h, 17% remained small and condensed, 59% were swollen with one nucleus, 24% showed two nuclei, of which 16% had a germ tube emerging. At 5 h, 8% remained small and condensed, 12% were swollen with one nucleus, 80% showed two nuclei, and 75% had a germ tube emerging. Finally, at 6 h, 8% remained small and condensed, probably reflecting non-living spores, 8% remained swollen with one nucleus, with the rest (84%) showing at least two nuclei and a germ tube. Thus, germination, defined as the breaking of dormancy, is cytologically visible between 1 and 2 h of growth in these conditions. Nuclear division clearly starts after 2 h of growth. Germ tube emergence is visible as early as after 3 h of growth but is significant between 4 and 5 h after inoculation. No significant differences were observed among strains carrying loss-of-function mutations in genes involved in purine transport (*uapA24*, *uapC201/401*, *azgA4*), vitamin biosynthesis (*biA1*, *pabaA1*, *pantoB100*, *pyroA4*, *riboD5*) or conidiospore pigment biosynthesis (*yA2*) (see *Experimental procedures* and Table 1). Figure 1C shows representative conidiospores after different germination time periods. Representative conidiospores were selected based on how a significant majority of conidiospores appeared under the microscope. Based on these observations, 1–2 h germinating conidiospores can be considered to be in the isotropic growth phase, and before the first nuclear division.

Purine uptake studies and Northern blot analyses were carried out in the above germination stages (Fig. 1). Uptake studies were carried out in strains expressing one functional purine transporter gene (*uapA*$^+$ *uapC*$^-$ *azgA*$^-$, *azgA*$^+$ *uapA*$^-$ *uapC*$^-$ and *uapC*$^+$ *uapA*$^-$ *azgA*$^-$). Uptakes were also carried out in a strain lacking all three major purine activities, that is a *uapA*$^-$ *uapC*$^-$ *azgA*$^-$ triple mutant (for full genotypes see Table 1 and *Experimental procedures*). This strain shows very low levels of purine uptake probably because of minor genetically unidentified transporters. Uptake measurements shown in this work correspond to values corrected by subtracting the background values obtained, under similar conditions, with the triple mutant strain.

Our results showed that *uapA*, *uapC* and *azgA* mRNA steady-state levels were undetectable in resting conidiospores and that their transcription is activated during the isotropic growth phase of conidiospores. This was in agreement with the observation that resting conidiospores lack any significant purine uptake activity and that UapA, UapC and AzgA activities develop rapidly upon germination. The requirement for *de novo* synthesis of purine transporters was tested further using cycloheximide, an inhibitor of translation. Cycloheximide completely abolished purine transport (see Fig. 1) and resulted in a total block of germination, with conidiospores and nuclei remaining condensed (Osherov and May, 2000). Actinomycin D, an inhibitor of mRNA synthesis, did not however inhibit the transcription of *azgA* or actin genes. As this may result from defective uptake in germinating conidia, this negative result is not significant (not shown).

The kinetics of purine transporter mRNA appearance and purine uptake activities were similar, but not identical, for the three genes. *azgA* transcription was already significant after 1 h of germination, reached a maximum at 2 h, and then slowly and progressively dropped to a somewhat lower level in the terminal stages of germination. This was in very good agreement with the kinetics of AzgA activity considering a 1 h delay between transcription and protein expression. *uapC* transcription was also evident after 1 h of germination, but mRNA steady-state levels reached a maximum after only 2 h of germination. Then, similar to *azgA*, *uapC* mRNA levels dropped to a somewhat lower level in the terminal stages of germination. Unlike the slow drop in AzgA activity at the later stages of germination, UapC activity remained fairly constant. Finally, *uapA*

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

Strain	Relevant genotype and characteristics	Reference
Wild type	*biA1*, standard wild-type strain, auxotroph for biotin	
uapA$^+$*uapC*$^-$*azgA*$^-$	*uapC401/201azgA4 pantoB100 biA1 yA2*, loss-of-function in *azgA* and *uapC*, auxotroph for pantothenic acid and biotin, yellow conidia	Diallinas *et al*. (1995)
uapA$^-$*uapC*$^+$*azgA*$^-$	*uapA24 azgA4 biA1*, loss-of-function in *uapA* and *azgA*, auxotroph for biotin	Diallinas *et al*. (1995)
uapA$^-$*uapC*$^-$*azgA*$^+$	*uapA24 uapC401/201 biA1*, loss-of-function in *uapA* and *uapC*, auxotroph for biotin	Diallinas *et al*. (1995)
uapA$^-$*uapC*$^-$*azgA*$^-$	*uapA24 uapC401/201 azgA4 pabaA1*, loss-of-function in *uapA*, *uapC* and *azgA*, auxotroph for *p*-aminobenzoic acid	Diallinas *et al*. (1995)
uaY$^-$	*uaY9 riboD5*, loss-of-function mutation in *uaY*, auxotroph for riboflavin	Suarez *et al*. (1995)
uaY^c	*uaY*^{c}*462 pyroA4 wA3*, constitutive mutation in *uaY*, auxotroph for pyridoxine, white conidia	Oestreicher and Scazzocchio (1995)
areA$^-$	*areA600 pabaA1 biA1*, loss-of-function mutation *areA*, auxotroph for *p*-ami nobenzoic acid and biotin	Ravagnani *et al*. (1997)
areA102	*areA102 pyroA4 fwA1*, abolishes binding of AreA in its targets in the 5′ regulator regions of *uapA* and *uapC*, auxotroph for pyridoxine, fawn conidia	Ravagnani *et al*. (1997)

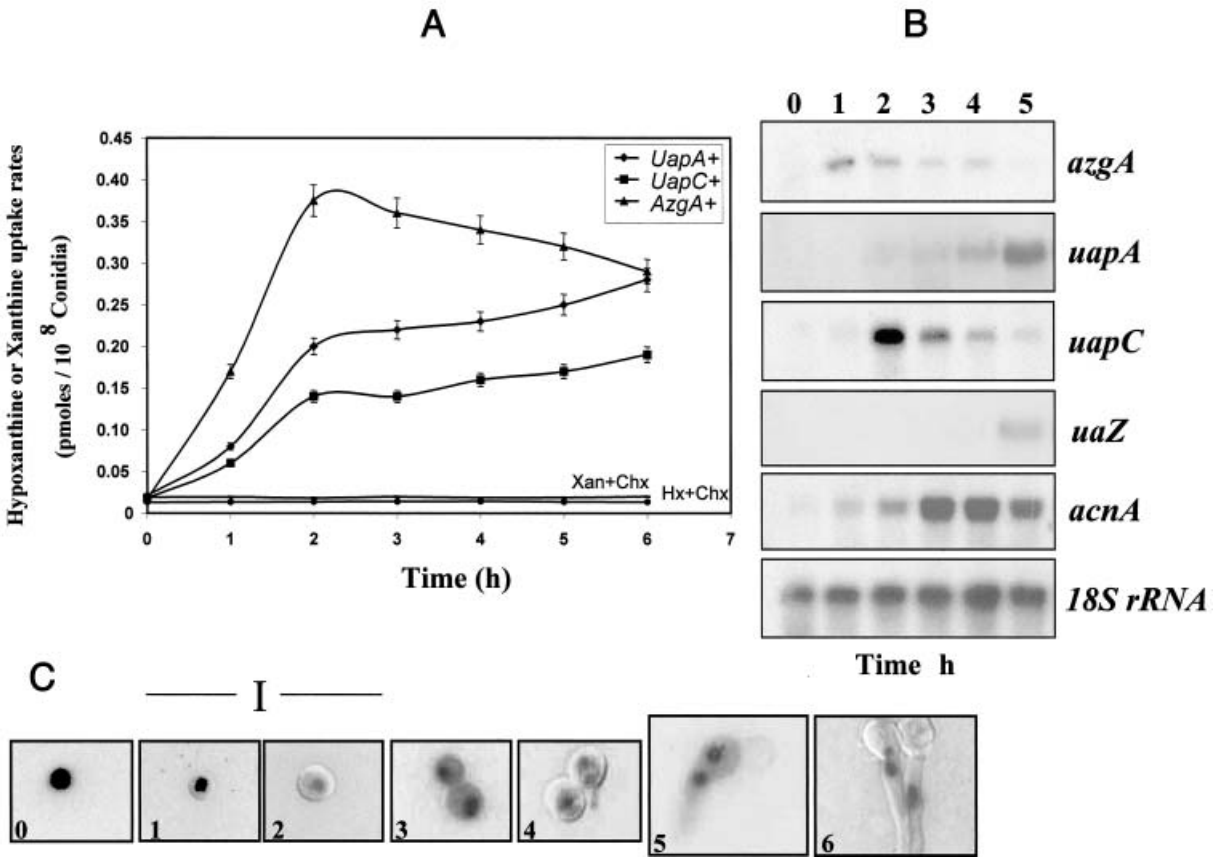

Fig. 1. Expression of purine transporters during conidiospore germination. Here, and in all subsequent figures, UapA and UapC activities were measured by estimating initial uptake rates of [^{3}H]-xanthine, whereas AzgA activity was measured by estimating initial uptake rates of [^{3}H]-hypoxanthine.
A. ^{3}H-radiolabelled xanthine (*uapA*$^+$*uapC*$^-$*azgA*$^-$ and *uapC*$^+$*uapA*$^-$*azgA*$^-$) or hypoxanthine (*azgA*$^+$*uapA*$^-$*uapC*$^-$) uptake rates in resting (0 h) and germinating (1–6 h) conidiospores in MM supplemented with urea as sole N source. Uptake of xanthine (Xan) or hypoxanthine (Hx) in a wild-type strain (*biA1*) germinating in urea in the presence of cycloheximide (Chx) is also shown as a control. Uptake measurements represent the averages of at least three independent experiments with standard deviations of <20%. For technical details, see *Experimental procedures*.
B. Northern blot analyses of *uapA*, *uapC* and *azgA* mRNA steady-state levels from resting (0 h) and germinating (1–5 h) conidiospores of a wild-type strain grown in MM supplemented with urea as sole N source. The Northern blot hybridized to a *uapA*-specific probe was exposed five times longer than the rest in order to detect better the low levels of *uapA*. Northern blot analyses of uric acid oxidase (*uaZ*) and actin (*acnA*) mRNA and 18S rRNA steady-state levels are also shown as controls (see *Experimental procedures* and *Results* for more details).
C. Nuclei staining of conidiospores from a wild-type strain (*biA1*), at different germination stages, as these were defined in the text and corresponding to the stages in which uptakes (A) and Northern blots (B) were performed. I stands for isotropic growth phase (see also *Experimental procedures*).

mRNA appearance was evident after 2 h of germination and continued to increase until the onset of germ tube appearance. This was in total agreement with the appearance of UapA transport activity. Taken together, the results presented above strongly suggested that the transcription of purine transporter genes was elicited during the isotropic growth phase of germination.

The kinetics of mRNA appearance of purine transporter genes was different from that of actin and *uaZ* mRNA (see Fig. 1B). Actin mRNA was very low but detectable in resting conidiospores, remained relatively low for 2 h of germination and increased dramatically after 3–5 h of germination. *uaZ*, the gene encoding uricase, an enzyme essential in purine catabolism (Oestreicher and Scazzocchio, 1993), and which, in mycelium, is co-regulated with purine transporter genes, was virtually not expressed during germination. Its mRNA became apparent at a stage coincident with the onset of germ tube appearance. The significance of these observations is discussed later.

Purine transporter expression in the isotropic growth phase is independent of the nitrogen source present in the medium

In *A. nidulans*, urea is considered as a non-repressing nitrogen source, which allows basal level expression of genes involved in N metabolism (Cove, 1966; Scazzocchio and Darlington, 1968). We tested whether different N

sources affect purine transport expression during conidiospore germination. To this end, we first examined whether germination, within the limits of microscopic observation, was affected by N availability. Germination stages in the presence of ammonia were essentially identical to those described in the presence of urea and shown in Fig. 1C (results not shown). Under conditions of N starvation, conidiospores initially germinated normally, as judged by the timing of swelling and acquirement of adhesion properties. However, we observed a difference in late events after initial germination (compare Figs 1C and 3C). Germ tube budding became visible only after 4 h (3%), at 5 h was 5%, and at 6 h 10%. At 6 h, <1% of germlings had two nuclei. Mycelium development was dramatically slowed down. Longer branched hyphae appear after 24 h (not shown).

Figures 2 and 3 show the results from Northern blot analyses and purine uptake measurements, performed as described previously, but in which urea was replaced by ammonium or no nitrogen source respectively. In the presence of ammonium (Fig. 2B), transcription of all three purine transporter genes is still activated early, at a stage (1–2 h) coincident with the isotropic growth phase of conidiospores. The kinetics of appearance and the mRNA steady-state levels present small differences compared with those obtained in urea for the same stages (1–2 h). In particular, *azgA* transcription is activated slightly later, *uapC* transcription slightly earlier, and *uapA* levels were barely visible. Lower levels of mRNAs were obtained for all genes at later stages of germination (3–5 h). These results were confirmed by uptake measurements. The results in Fig. 2A showed that, initially (0–2 h of germination), purine transport activities reach levels comparable to those obtained in the presence of urea. At later stages of germination, all activities dropped significantly.

In the absence of any nitrogen source during germination, transcription of all three purine transporter genes was activated as early as 1 h after inoculation (Fig. 3B). The kinetics of *azgA* and *uapC* appearance were nearly identical, at these stages, with those in the presence of urea (compare 1–2 h in Figs 1B and 3B). Interestingly, *uapA* mRNA transcription is activated earlier and reaches higher levels under nitrogen starvation conditions compared with germination with urea, and especially with ammonium. mRNA steady-state levels of all three purine transporter genes dropped dramatically after 3 h of germination. These results were in very good agreement with uptake measurements (Fig. 3A). In the first 2 h, all three purine transport activities increased to levels similar to those obtained in the presence of urea or ammonium, and then (3–6 h) dropped rapidly. Compared with the activities obtained after 2 h of germination in the presence of nitrogen sources, AzgA activity was somewhat reduced (62%), UapC activity was more or less identical, while UapA activity was increased (125%).

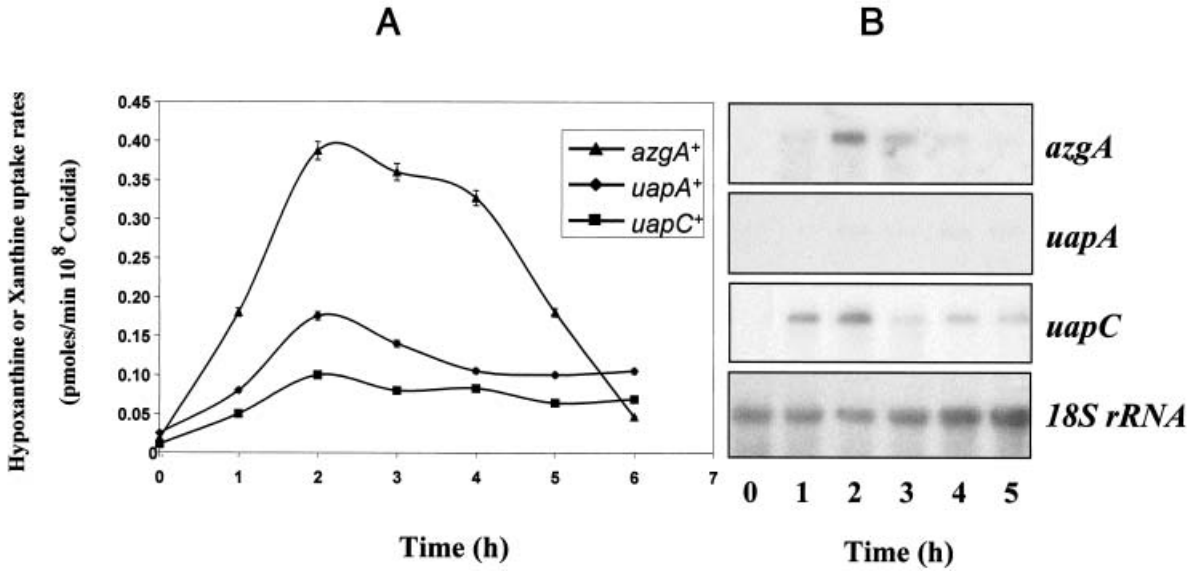

Fig. 2. Expression of purine transporters in conidiospores germinating in the presence of ammonia.
A. ^{3}H-radiolabelled xanthine (*uapA*$^+$*uapC*$^-$*azgA*$^-$ and *uapC*$^+$*uapA*$^-$*azgA*$^-$) or hypoxanthine (*azgA*$^+$*uapA*$^-$*uapC*$^-$) uptake rates in resting (0 h) and germinating (1–6 h) conidiospores in MM supplemented with 10 mM ammonium chloride as the sole N source. Uptake measurements represent the averages of at least three independent experiments with standard deviations of <20%.
B. Northern blot analyses of *uapA*, *uapC* and *azgA* mRNA steady-state levels from resting (0 h) and germinating (1–5 h) conidiospores of the wild-type strain grown in MM supplemented with 10 mM ammonium chloride as the sole N source. 18S rRNA steady-state levels are shown as a control.

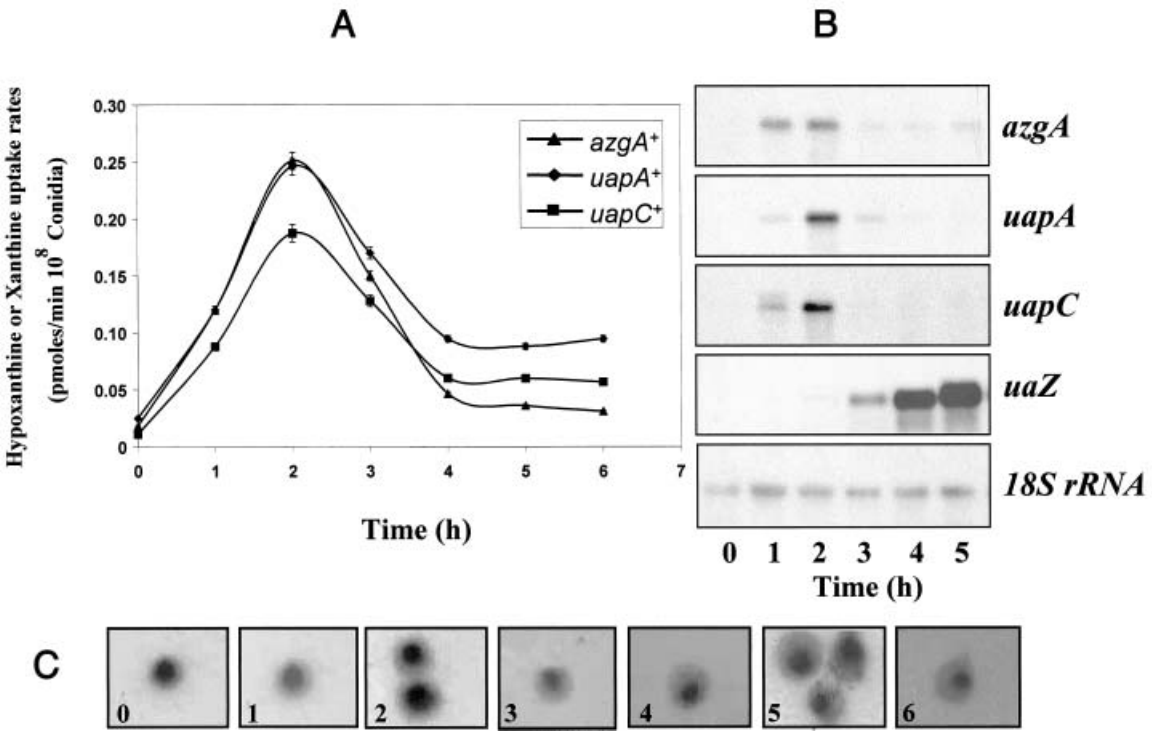

Fig. 3. Expression of purine transporters in conidiospores germinating under nitrogen starvation.
A. ^{3}H-radiolabelled xanthine (*uapA*$^{+}$*uapC*$^{-}$*azgA*$^{-}$ and *uapC*$^{+}$*uapA*$^{-}$*azgA*$^{-}$) or hypoxanthine (*azgA*$^{+}$*uapA*$^{-}$*uapC*$^{-}$) uptake rates in resting (0 h) and germinating (1–6 h) conidiospores in MM without a N source. Uptake measurements represent the averages of at least three independent experiments with standard deviations of <20%.
B. Northern blot analyses of *uapA*, *uapC*, *azgA* and *uaZ* mRNA steady-state levels from resting (0 h) and germinating (1–5 h) conidiospores of the wild-type strain grown in MM without a nitrogen source. 18S rRNA steady-state levels are shown as a control.
C. Nuclei staining of conidiospores from a wild-type strain (*biA1*), at different germination stages, as these appear under N starvation (see text).

We also examined the effect of N starvation on *uaZ* transcription. *uaZ* mRNA appeared after 3 h of germination but reached significant levels 4–5 h after inoculation (see Fig. 3B). Thus, although N starvation leads to activation of *uaZ* transcription compared with conditions in which a nitrogen source is present (see Fig. 1B), this occurs at a stage clearly different from that of the activation of purine transporter genes.

These results demonstrate that the activation of transcription of purine transporter genes is triggered, independently of the N status of the growth medium, very early during germination, at a stage coincident with the isotropic growth phase of conidiospores, and independently of later events, such as nuclear division or germ tube emergence.

The presence of purines does not affect the activation of purine transporter expression during the isotropic growth phase

In mycelium, all purine transporter genes are induced in the presence of 0.5 mM adenine, hypoxanthine or uric acid, or in the presence of 30–200 μM of the gratuitous inducer 2-thiouric acid (Scazzocchio and Darlington, 1968; Cecchetto *et al.*, 2004). We examined at what stage during germination conidiospores develop the capacity for purine induction of *uapA*, *uapC* and *azgA* genes, and whether purine induction and activation during germination are additive. Germination in the presence of purines was monitored microscopically and found to be essentially identical to germination in the presence of urea or ammonia (results not shown).

Results shown in Fig. 4A demonstrate that, on the whole, purine uptake activities do not increase significantly when germination takes place in the presence of purines. Similar results were obtained for *uapA*$^{+}$ and *uapC*$^{+}$ strains when 2-thiouric acid was used as an inducer, or with a wild-type *uapA*$^{+}$ *uapC*$^{+}$ *azgA*$^{+}$ strain (results not shown). Compared with the activities measured under non-inducing conditions (Fig. 1A), only AzgA showed an increase after the isotropic growth phase and the first nuclear division (3–6 h). Northern blot analysis (Fig. 4B) showed that purine induction becomes evident at the time of germ tube emergence (5 h). This is more evident for *azgA* and *uapA* than for *uapC* transcription.

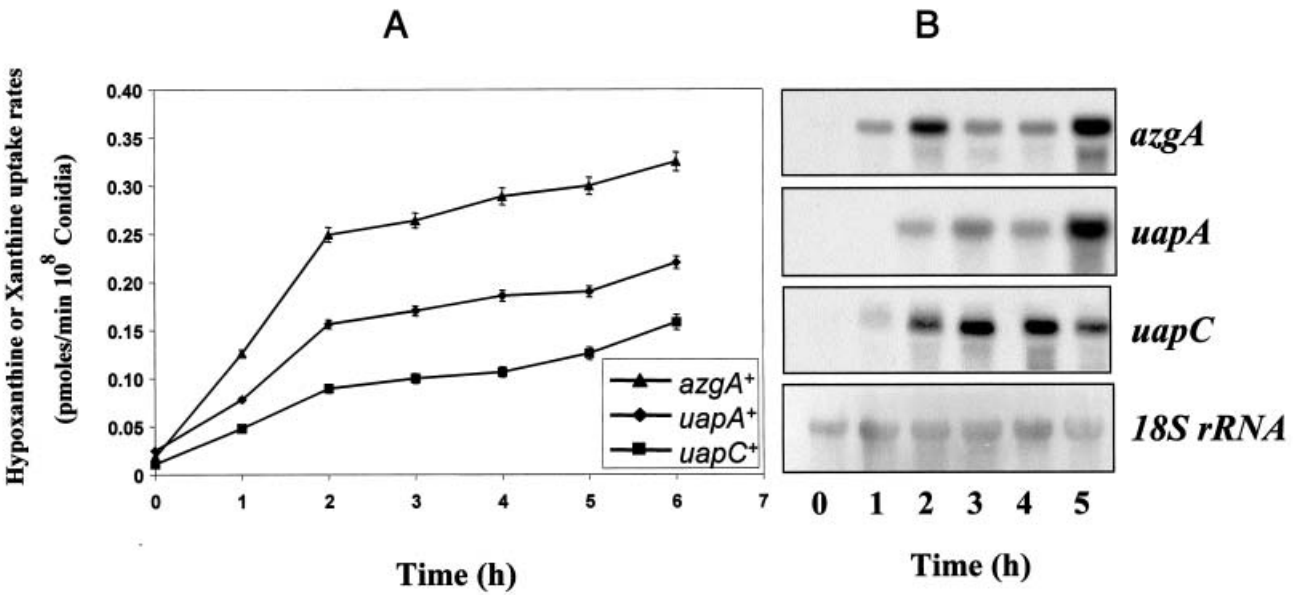

Fig. 4. Expression of purine transporters in conidiospores germinating in the presence of purines.
A. ^{3}H-radiolabelled xanthine (*uapA*$^+$*uapC*$^-$*azgA*$^-$ and *uapC*$^+$*uapA*$^-$*azgA*$^-$) or hypoxanthine (*azgA*$^+$*uapA*$^-$*uapC*$^-$) uptake rates in resting (0 h) and germinating (1–6 h) conidiospores in MM supplemented with 5 mM urea and 0.1 mg ml^{-1} either uric acid (for *uapA*$^+$*uapC*$^-$*azgA*$^-$ and *uapC*$^+$*uapA*$^-$*azgA*$^-$) or adenine (for *azgA*$^+$*uapA*$^-$*uapC*$^-$) (see *Experimental procedures* for the rationale of using uric acid or adenine for induction). Uptake measurements represent the averages of at least three independent experiments with standard deviations of <20%.
B. Northern blot analyses of *uapA*, *uapC* and *azgA* mRNA steady-state levels from resting (0 h) and germinating (1–5 h) conidiospores of the wild-type strain grown in MM supplemented with 5 mM urea and 0.1 mg ml^{-1} uric acid. 18S rRNA steady-state levels are shown as a control.

Transcriptional activation of purine transporter genes during germination is independent of the presence of a carbon source

Omission of nitrogen or phosphate from growth medium has been shown previously not to affect germination significantly (Osherov and May, 2001). On the other hand, omission of the carbon source blocks germination, whereas conidiospores can germinate in a 2% glucose solution. In order to investigate whether a carbon source is necessary for purine transporter expression in the isotropic growth phase, we performed xanthine and hypoxanthine uptake studies (Fig. 5A) and Northern blot analyses (Fig. 5B) with a wild-type strain (*uapA*$^+$ *uapC*$^+$ *azgA*$^+$) germinating in media with neither an N nor a C source. We first monitored germination microscopically (see Fig. 5C). Conidiospores remained small, with no adhesion properties and with condensed single nuclei for at least 4 h. In 5 h and 6 h samples, 10% of conidiospores were moderately swollen, of which 1–2% show a germ tube appearing (not shown). Thus, in agreement with earlier reports (Osherov and May, 2000), growth in the

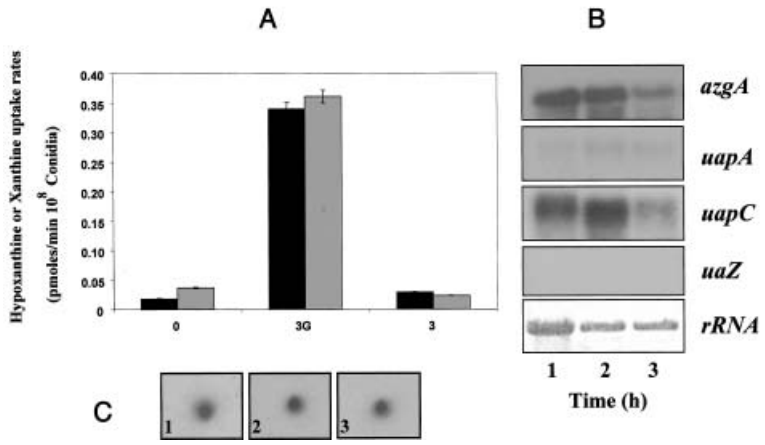

Fig. 5. Expression of purine transporters in conidiospores germinating in the absence of nitrogen and carbon sources.
A. ^{3}H-radiolabelled xanthine (light grey bar) or hypoxanthine (dark grey bar) uptake rates in resting (0 h) and germinating (3 h) conidiospores of the wild-type strain grown in MM with (3G) or without (3) a N (urea) and a C (glucose) source. Uptake measurements represent the averages of two independent experiments with standard deviations of <15%.
B. Northern blot analyses of *uapA*, *uapC*, *azgA* and *uaZ* mRNA steady-state levels from conidiospores germinating (1–3 h) in MM without either a N or a C source. rRNA levels are shown as a control.
C. Nuclei staining of conidiospores from a wild-type strain (*biA1*), after different germination times, as these appear under N and C starvation (see text).

absence of glucose led to conidiospores that remain small with condensed nuclei and without germ tubes emerging.

Figure 5A shows that, after 3 h of incubation, purine uptake activities were very low, similar to the levels detected in resting conidiospores. However, Fig. 5B shows that the expression of *azgA* and *uapC* remains substantial under these conditions. The expression of *uapA* is inhibited but not eliminated and possibly peaks at 2 h, similar to conditions of nitrogen starvation (Fig. 3). No mRNAs were detected at later stages (not shown). In contrast, no *uaZ*-specific mRNA was detected under the same conditions (see Fig. 5B)

Expression of purine transporter genes in the isotropic growth phase is independent of UaY

In mycelium, induction of the expression of all purine transporter genes is dependent on a functional *uaY* gene product (Gorfinkiel *et al*., 1993; Diallinas *et al*., 1995; Suarez *et al*., 1995; Cecchetto *et al*., 2004). We examined whether the expression during germination of purine transporter genes is dependent on the UaY regulator. Figure 6 shows uptake studies (Fig. 6A) and Northern blot analyses (Fig. 6B) performed in the loss-of-function mutant *uaY9* and the gain-of-function mutant *uaY^{c}462*. *uaY^{c}462* differentially affects the expression of different genes subject to UaY-mediated induction resulting in varied degrees of constitutivity and derepression (Oestreicher and Scazzocchio, 1995; Cecchetto *et al*., 2004). Neither of the two mutations affected germination stages (results not shown) as these were defined earlier for *uaY$^+$* (wild-type) strains and shown in Fig. 1C. Our results showed that *uapA*, *uapC* and *azgA* expression during germination is independent of the UaY transcriptional activator. Results from both uptake studies and Northern blot analyses using conidiospores from *uaY9* and *uaY^{c}462* were similar to results obtained in a *uaY$^+$* strain (see Fig. 1). It is noticeable that, in a *uaY* null mutation, the

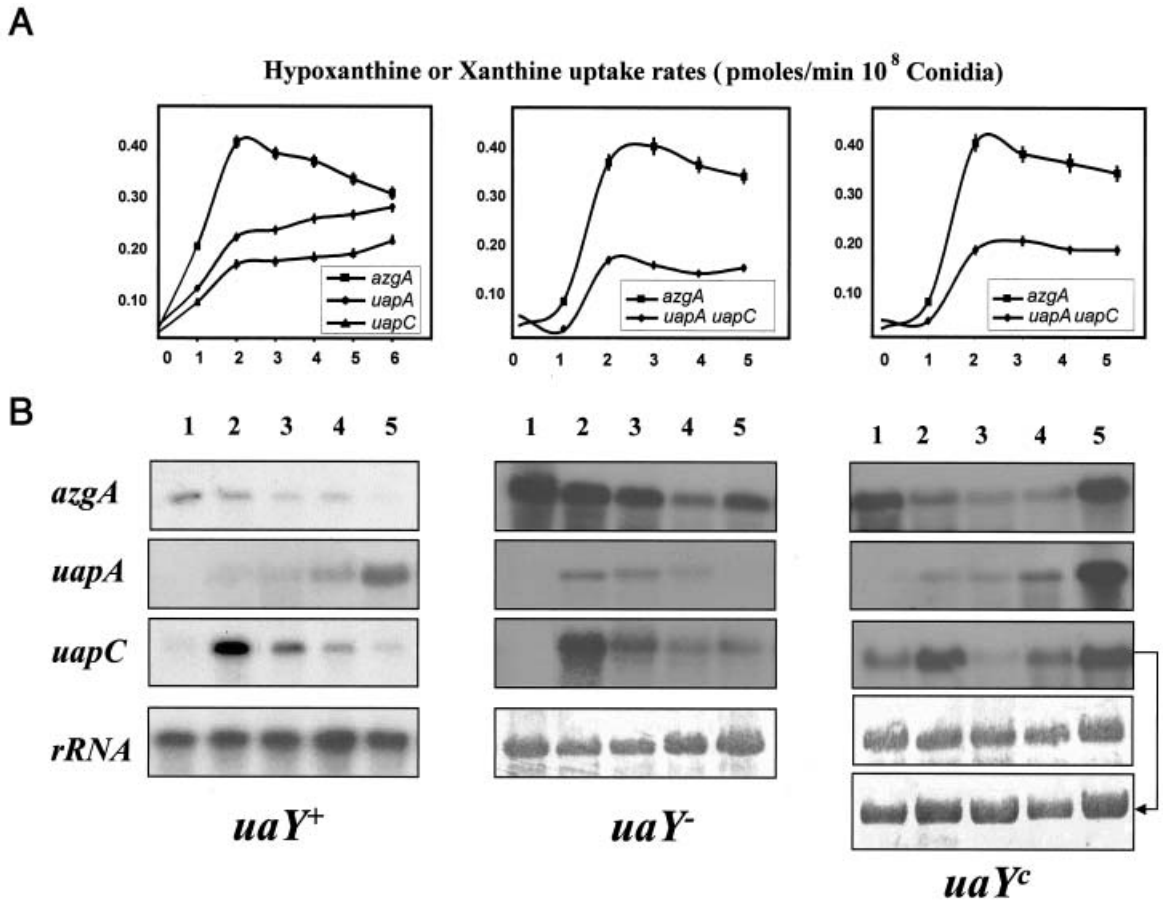

Fig. 6. Expression of purine transporters during germination is independent of UaY.
A. ^{3}H-radiolabelled xanthine or hypoxanthine uptake rates in resting (0 h) and germinating (1–5 h or 1–6 h) conidiospores of wild-type (*uaY$^+$*), *uaY$^-$* and *uaY^c* strains in MM supplemented with urea as sole N source. Uptake measurements represent the averages of three independent experiments with standard deviations of <20%.
B. Northern blot analyses of *uapA*, *uapC* and *azgA* mRNA steady-state levels from germinating (1–5 h) conidiospores of *uaY$^+$*, *uaY$^-$* and *uaY^c* strains grown in MM supplemented with urea as sole N source. rRNA levels or total RNA loading are also shown as a control.

uapA message peaks between 1 and 2 h, highlighting the fact that, in the isotropic stage of germination, *uapA* expression is independent of the specific transcription factor. That UaY takes over after the first mitosis and the emergence of the germinal tube is shown by the response of the three genes to the gain-of-function *uaYc462*, which reflects exactly what was described in mycelia (Oestreicher and Scazzocchio, 1995, Cecchetto *et al*., 2004).

Expression of purine transporter genes in the isotropic growth phase is differentially dependent on AreA

In mycelium, *areA* loss-of-function mutations lead to very low mRNA levels of all purine transporter genes under both inducing and non-inducing conditions. *uapC* is the only gene that conserves a significant basal level expression in *areA* loss-of-function mutants (Gorfinkiel *et al*., 1993). We examined the role of AreA on the expression of purine transporters during germination. Figure 7 shows uptake studies (Fig. 7A) and Northern blot analysis (Fig. 7B) performed in *areA102* and *areA600* mutants. *areA600* is a total loss-of-function mutation, whereas *areA102* is a mutation resulting in the inability of the AreA protein to bind to its target sequences in *uapA* (Ravagnani *et al*., 1997) and *uapC* (N. Oestreicher, G. Diallinas, M. V. Queiroz, D. Gomez and C. Scazzocchio, unpublished). *areA102* did not affect germination stages (results not shown) as these were defined earlier for *areA*$^+$ (wild-type) strains. *areA600* mutants can only be grown in MM containing ammonia as a N source (Arst and Cove, 1973; Ravagnani *et al*., 1997). The *areA600* strain used in this work showed a germination pattern essentially identical to that of an *areA*$^+$ (wild-type) strain also grown in ammonia (see Fig. 3C).

Our results showed that, although *azgA* mRNA steady-state levels in *areA* mutants were rather lower at 1 h compared with wild-type levels, *azgA* and *uapC* transcription was still activated during the isotropic

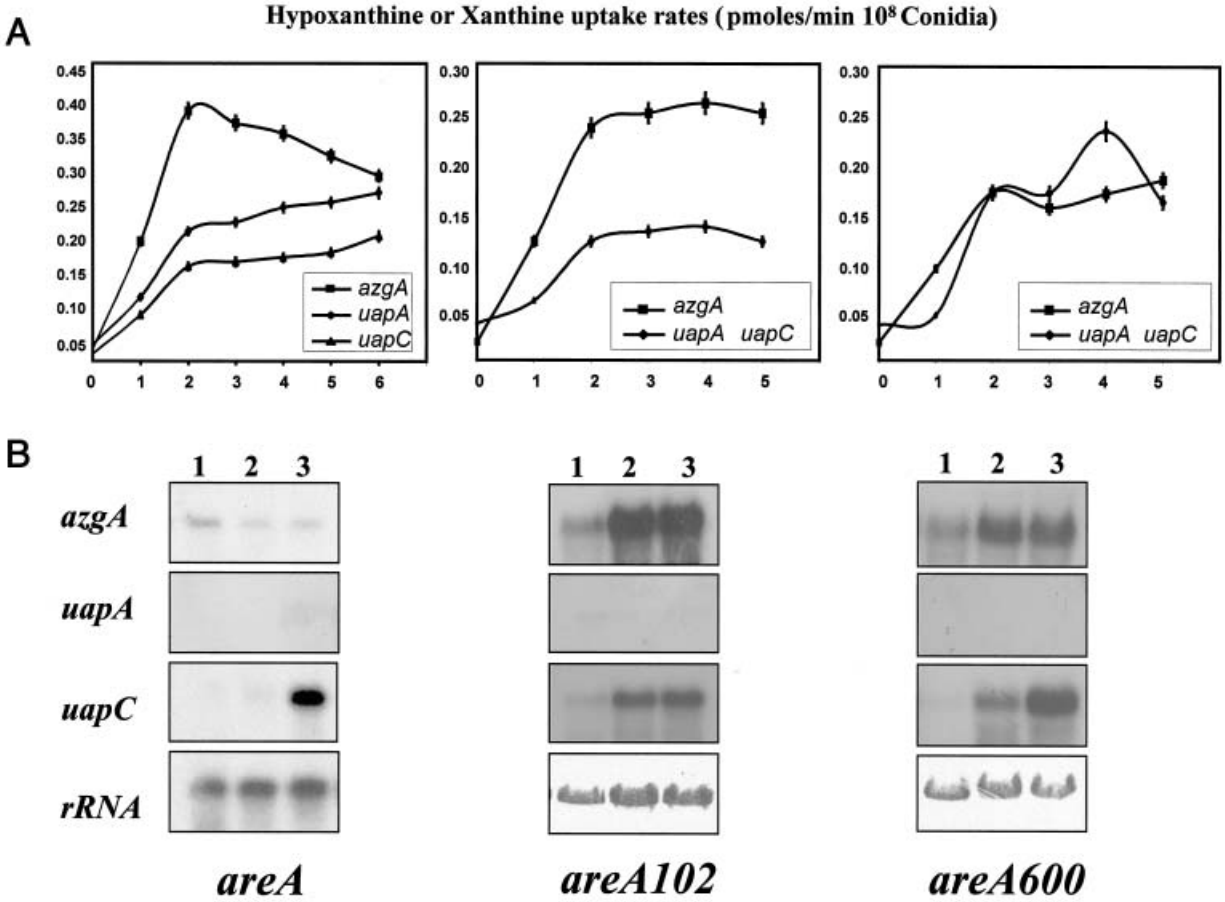

Fig. 7. Expression during germination of purine transporters is differentially dependent on AreA.
A. ^{3}H-radiolabelled xanthine or hypoxanthine uptake rates in resting (0 h) and germinating (1–5 h or 1–6 h) conidiospores of *areA*$^+$, *areA*$^-$ (*areA600*) and *areA102* strains in MM supplemented with urea as sole N source. Uptake measurements represent the averages of two independent experiments with standard deviations of <20%.
B. Northern blot analyses of *uapA*, *uapC* and *azgA* mRNA steady-state levels from germinating (1–3 h) conidiospores of *areA*$^+$, *areA*$^-$ (*areA600*) and *areA102* strains grown in MM supplemented with urea or ammonium, respectively, as sole N source. rRNA levels are also shown as a control.

growth phase. In contrast, no (*areA600*) or very low (*areA102*) *uapA* transcription could be detected in *areA* mutants. Thus, the xanthine uptake activity detected in uptake studies of *areA* mutants should be attributed to UapC.

Discussion

This work demonstrates that, in *A. nidulans* resting conidiospores, purine transporters are not expressed but, as soon as the spores come into contact with a medium that contains only salts and trace elements, *de novo* transcription of all three purine transporter genes is activated. This activation occurs during the isotropic growth phase of germination and independently of the timing of later events such as nuclear division and germ tube emergence. Transcriptional activation of *uapA*, *uapC* and *azgA* leads to the appearance of the corresponding purine transport activities if the germination medium also contains an energy supply, such as glucose. Despite the very low *uapA* transcript levels observed in urea, and especially in ammonia, uptake studies indicate that the transcription of this gene is also elicited during germination. The molecular mechanism underlying activation of purine transporter expression during germination is independent of UaY, the pathway-specific activator for purine catabolism. For at least *uapC* and *azgA*, this mechanism is also independent of AreA, the general GATA factor controlling nitrogen metabolism. The dependence of *uapA* expression on AreA is consistent with the very low levels of mRNA seen for this gene in the presence of ammonium. Thus, at least for *uapC* and *azgA*, their expression during germination is entirely independent of the mechanisms known to control expression in mycelia. We cannot exclude the possibility that a component of *uapA* expression might also be independent of AreA, but this could not be proved definitively because of the very low levels of *uapA* transcript levels in general and the lack of a triple $uapA^{+}$ $uapC^{-}$ $azgA^{-}$ $areA^{-}$ mutant strain.

Both regulatory systems known to control *uapA*, *uapC and azgA* transcription in mycelia, that is nitrogen metabolite repression and purine induction, are operational at later stages coincident with the onset of germ tube emergence (see Figs 2, 4, 6 and 7) but, as stated above, nitrogen repression affects *uapA* even at earlier stages of germination. Nitrogen starvation has a complex effect on the different genes examined in this work. During the isotropic growth phase, it has no effect on *uapC* and *azgA*, or on *uaZ*, actin and 18S rRNA expression, but activates *uapA* transcription, which is in line with the sensitivity of this gene to nitrogen metabolite repression. Interestingly, prolonged N starvation (>3 h) has a negative effect on the expression of all purine transporter genes. This may involve a mechanism analogous to that described in *Saccharomyces cerevisiae*, in which N starvation is perceived as a stress signal that activates a transporter degradation system involving ubiquitination and endocytosis (Galán *et al*., 1994; Omura *et al*., 2001; Krampe and Boles, 2002; Lucero *et al*., 2002).

Work presented here and previous results from us and from others (see *Introduction*) support the idea that germination activates the expression of solute transporters in *A. nidulans*. There is now evidence that the three purine transporters studied here and transporters for proline, aspartate/glutamate, nitrate, ammonium and uracil (Cook and Anthony, 1978; Brownlee and Arst, 1983; Tazebay *et al*., 1997; A. Apostolaki, C. Scazzocchio S. Amillis and G. Diallinas, unpublished) are activated very early during germination. Interestingly, transcription of non-transporter genes involved in purine (*uaZ*) or proline (*prnC* and *prnD*) utilization is not activated during germination. In addition, transporter mRNA steady-state levels reach their maxima even before a housekeeping protein such as actin. Furthermore, although we have only looked at a single non-transporter gene (*uaZ*), the independence of transcription from the presence of a C source seems to be specific for transporter genes. It thus seems that, in the repertoire of biochemical processes that become highly active very early during the isotropic growth phase (Bainbridge, 1971; D'Enfert, 1997), the expression of transporters, via an unidentified controlling system, gains its distinctive position. Early, often transient, transporter activation may serve as a strategy of dormant conidiospores for sensing the 'quality' of the environment and accordingly adapting their global uptake systems. Thus, unlike *S. cerevisiae*, in which highly specific sensor proteins can activate true transporters (Iraqui *et al*., 1999; Forsberg and Ljungdahl, 2001), *A. nidulans* might use its transporters *per se* for both sensing the environment and for the bulk transport of solutes. The dissection of transporter gene transcriptional activation during the isotropic phase of conidial germination may in fact provide a system to investigate the very early events in this process.

Experimental procedures

Aspergillus nidulans *strains and media*

The *A. nidulans* strains used in this study are shown in Table 1. Mutations resulting in vitamin requirements or conidiospore colour referred to in Table 1 do not affect the expression of genes involved in purine uptake or metabolism. *A. nidulans* complete (CM) and minimal (MM) media and growth conditions were as described previously (Cove, 1966; Scazzocchio and Arst, 1978). Unless stated otherwise, carbon source was 1% (w/v) glucose, and nitrogen source was 5 mM urea or 10 mM ammonium chloride or 0.1 mg ml^{-1} purines (uric acid or adenine).

Northern blot analysis

Total RNA was isolated as described previously (Tazebay *et al*., 1997). Northern blot analysis was performed as described previously (Tazebay *et al*., 1997). Gene-specific ^{32}P-radiolabelled probes for *uapA*, *uapC*, *azgA*, *uaZ* or 18S rRNA were prepared by a standard random priming method using gene-specific polymerase chain reaction (PCR) fragments corresponding to the open reading frame (ORF) of the corresponding genes.

Purine transport assays

UapA and UapC activities were measured by estimating initial uptake rates of [^{3}H]-xanthine, while AzgA activity was measured by estimating initial uptake rates of [^{3}H]-hypoxanthine. [^{3}H]-xanthine or [^{3}H]-hypoxanthine uptake was assayed in resting conidiospores (0 h), germinating conidiospores (1–5 h) or young mycelium (6 h), at 37°C, essentially as described previously (Diallinas *et al*., 1995; Tazebay *et al*., 1997). For achieving purine induction conditions, strains carrying functional *uapA* or uapC genes were induced by uric acid, the proposed physiological inducer of the system, as described previously (Gorfinkiel *et al*., 1993; Diallinas *et al*., 1995). Strains carrying only a functional *azgA* gene were induced by adenine. We have shown recently that adenine and hypoxanthine can induce, equally well with uric acid, possibly via their oxidation to uric acid, all genes involved in purine uptake and catabolism (Cecchetto *et al*., 2004). Results from transport assays represent the averages of at least two different experiments. Initial velocities were corrected by subtracting background uptake values, measured in the triple *uapA*$^-$*uapC*$^-$*azgA*$^-$ mutant, which result from diffusion or as yet genetically undefined minor purine transporters present in all strains used in uptake assays. The errors given in figures are standard errors of the mean value. Radiolabelled [^{3}H (G)]-hypoxanthine (specific activity 13 Ci mmol^{-1}) was obtained from NEN. [8-^{3}H]-xanthine (specific activity 9.8 Ci mmol^{-1}) was obtained from Moravek Biochemicals.

Germination, nuclear staining and microscopy

To monitor germination stages and subsequent morphogenetic events, conidiospores from resting conidiospores or cultures growing for 1, 2, 3, 4, 5 or 6 h were collected and observed under an epifluorescence Axionplan Zeiss microscope, directly or after nuclei staining. Nuclear staining was carried out, according to the supplier's instructions (Molecular Probes), with Hoechst 33258 (bis-benzimide) and photographed using Kodak TMAX 400 film. Photographs shown in Figs 1C, 3C and 5C combine fluorescence with Nomarski. We have observed conidiospore germination under all conditions used in Northern blot analyses and uptake studies and for all strains used in this work. In all cases, and for each stage, 250–500 conidiospores were observed after nuclear staining. As described in the text, mutations in *uapA*, *uapC*, *azgA*, *uaY*, *areA* and *yA* had no effect on germination stages. Different vitamin auxotrophic mutations, and especially *biA1*, had a minor effect (30–45 min) on late events such as the timing of germ tube appearance. Germination stages were essentially identical when the N source was urea, ammonia or urea plus purines. In contrast, germination in the absence of a N source was severely delayed (see text and Fig. 3C), whereas in the absence of both a C and a N source, it was totally abolished (Fig. 5; results not shown).

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References

Amillis, S., Koukaki, M., and Diallinas, G. (2001) Substitution F569S converts UapA, a specific uric acid-xanthine transporter, into a broad specificity transporter for purine-related solutes. *J Mol Biol* **313:** 765–774.

Arst, H.N., Jr, and Cove, D. (1973) Nitrogen metabolite repression in *Aspergillus nidulans*. *Mol Gen Genet* **126:** 111–141.

Bainbridge, B.W. (1971) Macromolecular composition and nuclear division during spore germination in *Aspergillus nidulans*. *J Gen Microbiol* **66:** 319–325.

Brownlee, A.G., and Arst, H.N., Jr (1983) Nitrate uptake in *Aspergillus nidulans* and involvement of the third gene of the nitrate assimilation gene cluster. *J Bacteriol* **155:** 1138–1146.

Cecchetto, G., Amillis, S., Diallinas, G., Scazzocchio, C., and Drevet, C. (2004) The AzgA purine transporter of *Aspergillus nidulans*: characterization of a protein belonging to a new phylogenetic cluster. *J Biol Chem* (in press).

Cook, R., and Anthony, C. (1978) Regulation by glutamine of ammonia transport in *Aspergillus nidulans*. *Biochim Biophys Acta* **113:** 51–56.

Cove, D.J. (1966) The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. *Biochim Biophys Acta* **113:** 51–56.

D'Enfert, C. (1997) Fungal spore germination: insights from the molecular genetics of *Aspergillus nidulans* and *Neurospora crassa*. *Fung Genet Biol* **21:** 163–172.

Darlington, A.J., and Scazzocchio, C. (1967) Use of analogues and the substrate specificity of mutants in analysis of purine uptake and breakdown in *Aspergillus nidulans*. *J Bacteriol* **93:** 937–940.

De Koning, H., and Diallinas, G. (2000) Nucleobase transporters – review. *Mol Membr Biol* **17:** 75–94.

Diallinas, G., and Scazzocchio, C. (1989) A gene coding for the uric acid-xanthine permease of *Aspergillus nidulans*: inactivational cloning, characterization and sequence of a *cis*-acting mutation. *Genetics* **122:** 341–350.

Diallinas, G., Gorfinkiel, L., Arst, H.N., Jr, Cecchetto, G., and Scazzocchio, C. (1995) Genetic and molecular characterization of a gene encoding a wide specificity purine permease of *Aspergillus nidulans* reveals a novel family of

transporters conserved in prokaryotes and eukaryotes. *J Biol Chem* **270:** 8610–8622.

Diallinas, G., Valdez, J., Sophianopoulou, V., Rosa, A., and Scazzocchio, C. (1998) Chimeric protein analysis reveals a region involved in function and specificity of purine transporters in the filamentous fungus *Aspergillus nidulans* conserved in bacteria, plants and metazoan homologues. *EMBO J* **17:** 3827–3837.

Fillinger, S., Chaveroche, M.K., Shimizu, K., Keller, N., and d'Enfert, C. (2002) cAMP and *ras* signalling independently control spore germination in the filamentous fungus *Aspergillus nidulans*. *Mol Microbiol* **44:** 1001–1016.

Forsberg, H., and Ljungdahl, P.O. (2001) Sensors of extracellular nutrients in *Saccharomyces cerevisiae*. *Curr Genet* **40:** 91–109.

Galán, J.M., Volland, C., Urban-Grimal, D., and Haguenauer-Tsapis, R. (1994) The yeast plasma membrane uracil permease is stabilized against stress induced degradation by a point mutation in a cyclin-like 'destruction box'. *Biochem Biophys Res Commun* **201:** 769–775.

Gorfinkiel, L., Diallinas, G., and Scazzocchio, C. (1993) Sequence and regulation of the *uap*A gene encoding a uric acid-xanthine permease in the fungus *Aspergillus nidulans*. *J Biol Chem* **268:** 23376–23381.

Iraqui, I., Vissers, S., Bernard, F., de Craene, J.O., Boles, E., Urrestarazu, A., and Andre, B. (1999) Amino acid signalling in *Saccharomyces cerevisiae*: a permease-like sensor of external amino acids and F-Box protein Grr1p are required for transcriptional induction of the AGP1 gene, which encodes a broad-specificity amino acid permease. *Mol Cell Biol* **19:** 989–1001.

Krampe, S., and Boles, E. (2002) Starvation-induced degradation of yeast hexose transporter Hxt7p is dependent on endocytosis, autophagy and the terminal sequences of the permease. *FEBS Lett* **513:** 193–196.

Kudla, B., Caddick, M.X., Langdon, T., Martinez-Rossi, N.M., Bennett, C.F., Sibley, S., *et al.* (1990) The regulatory gene *areA* mediating nitrogen metabolite repression in *Aspergillus nidulans*. Mutations affecting specificity of gene activation alter a loop residue of a putative zinc finger. *EMBO J* **9:** 1355–1364.

Lucero, P., Moreno, E., and Lagunas, R. (2002) Catabolite inactivation of the sugar transporters in *Saccharomyces cerevisiae* is inhibited by the presence of a nitrogen source. *FEM Yeast Res* **1:** 307–314.

Meintanis, C., Karagouni, A.D., and Diallinas, G. (2000) Amino acids residues N^{450} and Q^{449} are critical for the uptake capacity and specificity of UapA, a prototype of a universally conserved nucleobase-ascorbate transporter family. *Mol Membr Biol* **17:** 47–58.

Momany, M. (2002) Polarity in filamentous fungi: establishment, maintenance and new axes. *Curr Opin Microbiol* **5:** 580–585.

Morozov, I.Y., Galbis-Martinez, M., Jones, M.G., and Caddick, M.X. (2001) Characterization of nitrogen metabolite signalling in *Aspergillus* via the regulated degradation of areA mRNA. *Mol Microbiol* **42:** 269–277.

Oestreicher, N., and Scazzocchio, C. (1993) Sequence, regulation, and mutational analysis of the gene encoding urate oxidase in *Aspergillus nidulans*. *J Biol Chem* **268:** 23382–23389.

Oestreicher, N., and Scazzocchio, C. (1995) A single amino acid change in a pathway-specific transcription factor results in differing degrees of constitutivity, hyperinducibility and derepression of several structural genes. *J Mol Biol* **249:** 693–699.

Omura, F., Kodama, Y., and Ashikari, T. (2001) The N-terminal domain of the yeast permease Bap2p plays a role in its degradation. *Biochem Biophys Res Commun* **287:** 1045–1050.

Osherov, N., and May, G.S. (2000) Conidial germination in *Aspergillus nidulans* requires RAS signaling and protein synthesis. *Genetics* **155:** 647–656.

Osherov, N., and May, G.S. (2001) The molecular mechanisms of conidial germination. *FEMS Microbiol Lett* **199:** 153–160.

Ravagnani, A., Gorfinkiel, L., Langdon, T., Diallinas, G., Adjaj, E., Demais, S., *et al.* (1997) Subtle hydrophobic interactions between the seventh residue of the zinc finger loop and the first base of an HGATAR sequence determine promoter-specific recognition by the *Aspergillus nidulans* GATA factor AreA. *EMBO J* **16:** 3974–3986.

Scazzocchio, C., and Arst, H.N., Jr (1978) The nature of an initiator constitutive mutation in *Aspergillus nidulans*. *Nature* **274:** 177–179.

Scazzocchio, C., and Darlington, A.J. (1968) The induction and repression of the enzymes of purine breakdown in *Aspergillus nidulans*. *Biochim Biophys Acta* **166:** 557–568.

Scazzocchio, C., Sdrin, N., and Ong, G. (1982) Positive regulation in a eukaryote, a study of the uaY gene of *Aspergillus nidulans*. I. Characterization of alleles, dominance and complementation studies, and a fine structure map of the *uaY-oxpA* cluster. *Genetics* **100:** 185–208.

Suarez, T., Vieira de Quieroz, M., Oestreicher, N., and Scazzocchio, C. (1995) The sequence and binding specificity of UaY, the specific regulator of the purine utilization pathway in *Aspergillus nidulans*, suggests an evolutionary relationship with the PPR1 protein of *Saccharomyces cerevisiae*. *EMBO J* **14:** 1453–1467.

Tazebay, U.H., Sophianopoulou, V., Cubero, B., Scazzocchio, C., and Diallinas, G. (1995) Post-transcriptional control and kinetic characterization of proline transport in germinating conidiospores of *Aspergillus nidulans*. *FEMS Microbiol Lett* **132:** 27–37.

Tazebay, U.H., Sophianopoulou, V., Scazzocchio, C., and Diallinas, G. (1997) The gene encoding the major proline transporter of *Aspergillus nidulans* is upregulated during conidiospore germination and in response to proline induction and amino acid starvation. *Mol Microbiol* **24:** 105–117.

Wilson, R.A., and Arst, H.N., Jr (1998) Mutational analysis of AREA, a transcriptional activator mediating nitrogen metabolite repression in Aspergillus nidulans and a member of the 'streetwise' GATA family of transcription factors. *Microbiol Mol Biol Rev* **62:** 586–596.