

Nutrient transport into germinating *Trichoderma atroviride* conidia and development of its driving force

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The exit from dormancy and the start of growth should be preceded or at least accompanied by the uptake of nutrients. In this work we studied changes in the transport of several nutrients into *Trichoderma atroviride* conidia. Germination started with a short period of isodiametric growth (conidial swelling), followed by polarized growth (germ tube formation) after about 8 h at 26 °C. The onset of isodiametric growth required the presence of external both phosphate and nitrate. At the same time, an increased uptake of precursors of macromolecules and phospholipids (¹⁴C- or ³H-labelled valine, uracil, *N*-acetylglucosamine and choline) occurred. A low uptake of these precursors was observed also in non-germinating conidia. Concomitantly, this uptake developed an increased sensitivity to the uncoupler 3,3',4',5-tetrachlorosalicylanilide. Expression and activity of H⁺-ATPase started after completing isodiametric growth, suggesting that the proton-motive force (PMF) generated by H⁺-ATPase may be an accelerator of nutrient uptake and metabolism. ¹⁴C-valine uptake was also measured into a mutant with disrupted *pma1* gene. This mutant did not form conidia. The mutant also exhibited uncoupler sensitivity of ¹⁴C-valine uptake. These observations showed that a PMF must have been generated by a mechanism(s) other than the H⁺-ATPase activity in the WT before H⁺-ATPase expression and in mycelia with disrupted H⁺-ATPase.

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INTRODUCTION

Conidia of filamentous fungi are not only propagating structures but also enable survival under conditions unfavourable for growth. Conidia are viable for several tens of years as suggested from analyses of damaged culture heritage (Arroyo, 2009) but several observations suggest that conidia may be able to retain their viability for up to geological timescales (Abyzov *et al.*, 2001; Pokorný *et al.*, 2004). These observations indicate that conidia persist under unfavourable conditions in a state of minimal metabolic turnover (such as the cryptobiotic state: Clegg, 2001), and germinate upon transition to favourable conditions. Thus, the germination of conidia could represent a model for the transition from a cryptobiotic (dormant) to a growing state.

The breaking of dormancy is the most mysterious part of conidial germination and its nature remains essentially

unknown. This step is followed by conidial swelling, formation of the germ tube and maintenance of hyphal growth (Oshero & May, 2001; Harris & Momany, 2004).

There are several prerequisites for fungal spore germination that may be considered universal: water (or high humidity), O₂ and CO₂, and suitable temperature (Carlile *et al.*, 2001). This contrasts with nutritional requirements, which are variable among fungal species (Abdel-Rahim & Arbab, 1985; Gachomo *et al.*, 2009). Processes that accompany the germination of conidia and subsequent changes in morphology are well described (for reviews see Schmit & Brody, 1976; d'Enfert 1997; Carlile *et al.*, 2001). Knowledge of the mechanisms underlying genetic and metabolic changes during spore germination in several fungal species has largely come in recent decades, including osmolyte metabolism (d'Enfert *et al.*, 1999; Döhlemann *et al.*, 2006), protein synthesis (Leng *et al.*, 2008; Oh *et al.*, 2010; El-Akhal *et al.*, 2013), changes of gene expression (Liu *et al.*, 2007; Lamarre *et al.*, 2008; Novodvorska *et al.*, 2013; van Leeuwen *et al.*, 2013) or involvement of signalling pathways (Oshero & May, 2001; d'Enfert, 1997; Mukherjee *et al.*, 2007; Eaton *et al.*, 2012).

Abbreviations: CHOL, [methyl-³H]choline chloride; L-Val, L-[U-¹⁴C]valine; NAG, *N*-acetyl-D-[1-³H]glucosamine; PMF, proton-motive force; RT-PCR, reverse transcriptase PCR; TCS, 3,3',4',5-tetrachlorosalicylanilide; URA, [2-¹⁴C]uracil.

Metabolism of dormant conidia lacks respiratory activity and may be supported by fermentative metabolism (Teutschbein *et al.*, 2010). Their germination is accompanied by the onset of cytochrome *c* oxidase biosynthesis (Hawley & Greenawalt, 1975; Brambl, 1980; Stade & Brambl, 1981) and of respiration (Chovanec *et al.*, 2005; Nižňanský *et al.*, 2013), the latter being indispensable for germination (Liu *et al.*, 2007; Taubitz *et al.*, 2007).

It is feasible that the germination of conidia in a favourable environment begins with the uptake of nutrients necessary to supply material and energy for development of the fungus, as endogenous carbon and nitrogen resources are not sufficient for germination (Palmero Llamas *et al.*, 2008), although germination could be activated by metabolite sensing mechanism(s) without a transport step (Hayer *et al.*, 2013, 2014). Several studies have investigated transport during conidia germination. Nucleoside and nucleobase uptake and its underlying mechanisms were studied in conidia of *Neurospora crassa* (Schiltz & Terry, 1970; Magill & Magill, 1975; Pendyala & Wellman, 1977; Greer & Wellman, 1981). The transport of purines into germinating *Aspergillus niger* conidia and its genetic background were studied by Amillis *et al.* (2004) who showed that three genes for purine transporters are expressed during conidia germination without induction by external purines, and with a concomitant increase in purine uptake. The fourth purine transporter that could partially compensate for the loss of all other purine transporter genes was identified by Vlanti & Diallinas (2008). Transports of amino acids into conidia of *N. crassa* (Tisdale & DeBusk, 1970; Railey & Kinsey, 1976; DeBusk & DeBusk, 1980), arthrospores of *Geotrichum candidum* (McEvoy & Murray, 1972) and conidia of *Aspergillus oryzae* (Sinohara, 1974) or *A. niger* (Tazebay *et al.*, 1995; 1997) were also stimulated during germination, being mediated by multiple transport systems. Transport of saccharides was studied by Aitken & Niederpruem (1972).

An important aspect of nutrient transport into fungal hyphae relates to the nature of the driving force. Early observations indicated that uptake of amino acids (Kotyč & Říhová, 1972), choline (Robson *et al.*, 1992) or saccharides (Kotyč & Höfer, 1965; Deák & Kotyč, 1968) requires energy. Protons were shown to accompany amino acid transport (Eddy *et al.*, 1970; Eddy & Nowacki, 1971; Hillenga *et al.*, 1996). The discovery of an H⁺-ATPase in *N. crassa* (Scarborough, 1976) and in yeasts (Villalobo *et al.*, 1981) led to its establishment as the main source of the proton-motive force (PMF) required for uptake of nutrients (Goffeau & Slayman, 1981; Kotyč, 1983).

In *Trichoderma atroviride* we studied changes in marker enzymes of several organelles during ageing (Šimkovič *et al.*, 2007). Activity of H⁺-ATPase decreases progressively to virtually zero during ageing. We also found that conidia are formed in aged mycelia. This indicated that this enzyme is either not present or not active in conidia. This may have its origin in the observation of Tuduri *et al.* (1985) that

activity of this enzyme decreases during exponential growth of yeasts. Thus, H⁺-ATPase activity may be developmentally regulated.

In this work we studied the development of nutrient transport during *T. atroviride* conidia germination. Precursors of macromolecules and phospholipids were chosen as nutrients. The results confirm that H⁺-ATPase activity is negligible or absent in dormant conidia and appears only after the isodiametric phase of growth is completed. Furthermore, the result indicates that the H⁺-ATPase generates a sufficient driving force for mycelial growth. As a complement to the present study, changes of the metabolome were measured in germinating *T. atroviride* conidia, which demonstrated the activity and dynamics of metabolic pathways during germination (Kaliňák *et al.*, 2014).

METHODS

Strain. Strain CCM F-534 of *T. atroviride* (Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic) was used. The fungus was kept on the surface of agar slants with Czapek–Dox (CzD) medium supplemented with 0.5 % (w/v) yeast extract (YE).

Preparation of dormant and germinated conidia. Conidia from 2-week-old cultures grown at 27 °C were used for transport experiments. Five millilitres of sterile water was added to the surface of an agar slant and the conidia were carefully scraped off with a sterile needle. Conidial suspension was aseptically filtered through several layers of gauze, and washed three times with sterile water by centrifugation at 500 g for 10 min at room temperature. After the last centrifugation the conidial pellet was suspended in a minimal volume of water, filtered through sterile nylon mesh (43 µm pore diameter) to remove aggregates of conidia, and counted using a haemocytometer. Conidia were then used either directly for transport experiments (as dormant conidia) or for submerged cultivation to obtain conidia in different stages of germination. Submerged cultivation was performed in liquid CzD medium containing 0.5 % (w/v) YE (10⁷ conidia ml⁻¹) on a rotary shaker at 4 Hz, for the time indicated (0–50 h) at 27 °C. The germinated conidia were washed by centrifugation with abundant deionized water to remove the growth medium (three times at 1200 g for 5 min at room temperature), concentrated by centrifugation (as above) and immediately used for transport experiments.

During submerged cultivation, aliquots of conidial suspension were taken, filtered through pre-weighed membrane filters with a 0.6 µm pore size (Sartorius nitrocellulose filter) and used for dry mass determination (drying at 70 °C to constant weight). Simultaneously, aliquots were used for microscopic inspection (AxioImager A1; Carl Zeiss).

Transport experiments. For transport experiments under non-growing conditions, conidia were suspended in 1 % (w/v) NaCl buffered with 40 mM Tris/HCl, pH 7.4, to a final concentration of 5 × 10⁷ ml⁻¹. Liquid CzD medium without YE, with 40 mM Tris/HCl, pH 7.4, was used to suspend the conidia for transport experiments performed under growing conditions. Transport measurements were started by the addition of radioactive tracer solution to conidial suspension (final concentration 0.2 mM and specific activity 1500 c.p.m. nmol⁻¹), followed by fast vortexing and incubation with reciprocal shaking (100 r.p.m.) for the time indicated in the figures

(usually 20 min). Subsequently, aliquots of 0.5 ml were withdrawn and filtered through a nitrocellulose membrane filter (0.6 µm pore diameter; Sartorius) using a vacuum filtration apparatus. The conidia were washed twice with 3.5 ml of 40 mM Tris/HCl buffer, pH 7.4, containing 1 % (w/v) NaCl and used for radioactivity determination by liquid scintillation counting (1214 RackBeta; LKB Wallac). If not indicated otherwise, all experiments were performed at 25 °C. This procedure was used for the measurement of transport of ^{14}C -labelled and of ^3H -labelled metabolites: *N*-acetyl-D-[1- ^3H]glucosamine (NAG) (GE Healthcare), L-[U- ^{14}C]valine (L-Val), [2- ^{14}C]uracil (URA) and [methyl- ^3H]choline chloride (CHOL) (Amersham Bioscience). When the effect of inhibitors was tested, these were added 5 min prior to the addition of radionuclide. The experiments were performed in triplicate. The results are presented as mean \pm SE of a representative experiment, repeated three times independently.

Expression of the *pma1* gene. The expression profile of the *pma1* gene was determined by semi-quantitative reverse transcriptase PCR (RT-PCR). RNA was extracted from dormant conidia, and from conidia germinated for 5, 10 and 20 h according to Mukhtar *et al.* (1998). RNA was purified with an RNA cleanup kit (Qiagen). cDNA was synthesized by RT-PCR kit (5prime) using oligo(dT)₁₈ primer (Fermentas). Amplification of the *pma1* gene was carried out by means of specific primers, 5'-TCCGTTCTCTGGTGTG-3' (fwd) and 5'-AGCTCCCTGGAGGAAG-3' (rev). Actin was used as housekeeping gene and its cDNA was amplified with primers 5'-AGAGCTCCAGCTTGGAGAAGT-3' (fwd) and 5'-GAGCAAGAGC-AGTGATCTCCT-3' (rev). The PCR conditions for amplification of the two genes were as described by Olejníková *et al.* (2011). The RT-PCR products were analysed by gel electrophoresis on 1 % (w/v) agarose gels.

Construction of the $\Delta pma1$ mutant strain. $\Delta pma1$ mutant strains prepared by Olejníková *et al.* (2011) were used for experiments. These mutants grew slowly and were sensitive to fast shaking under submerged cultivation. Therefore, all measurements were made with cultures grown on solid medium.

Measurement of L-Val uptake into WT and $\Delta pma1$ mutant grown on solid medium. $\Delta pma1$ mutant and WT strains were cultivated on cellophane discs placed on the surface of modified CzD agar medium [supplemented with 0.5 % (w/v) casein hydrolysate, 0.5 % (w/v) peptone, 0.5 % (w/v) YE and 2 % (w/v) agar] for 5 days ($\Delta pma1$) or 3 days (WT) at room temperature in the dark (due to different horizontal growth rate of the two strains, the length of cultivation was normalized on the basis of culture diameter). After the cultivation was finished, cellophane discs with mycelium were placed on the polyamide net (43 µm pore diameter) and washed by filtration with 50 ml liquid CzD medium (without YE and any peptide/nitrogen source). Discs were then transferred to the surface of 5 ml of fresh liquid CzD medium (without YE) containing 200 µM L-Val (specific activity 2000 c.p.m. nmol⁻¹) and 40 mM Tris/HCl buffer, pH 7.4. Uptake of L-Val by both strains, $\Delta pma1$ and WT, was performed by static incubation of the mycelium with L-Val for 5 h, and stopped by filtration with 50 ml of liquid CzD medium. Mycelium collected was used for determination of radioactivity by liquid scintillation counting. When the effect of 3,3',4',5-tetrachlorosalicylanilide (TCS) (Eastman-Kodak) was measured, the protonophore was added to CzD medium together with ^{14}C -labelled L-Val. The experiments were performed in triplicate, and the results are presented as the mean \pm SE of a representative experiment, which was repeated twice for $\Delta pma1$ mutant and WT strains.

Simultaneously with the transport measurements, mycelia grown on the cellophane discs were taken, filtered through a pre-weighed glass-fibre membrane filter (Whatman GF) and used for dry mass determination (drying at 70 °C to constant weight).

Determination of vanadate-sensitive ATPase activity. Vanadate-sensitive ATPase activity, a marker enzyme for the fungal plasma membrane, was measured according to Serrano (1978) as modified by Olejníková *et al.* (2011). Aliquots of conidial homogenate containing 100 µg of protein were mixed with the assay medium containing: 25 mM MES-Tris (pH 6.5), 500 mM sorbitol, 5 mM NaN₃, 100 mM KNO₃, 0.2 mM Na₂MoO₄ and 5 mM MgSO₄. The volume was adjusted to 95 µl. The reaction was started by the addition of 5 µl ATP (Na₂ATP, 100 mM) and the assay mixture was incubated for 120 min at 37 °C. The reaction was stopped by the addition of 50 µl TCA (50 %, v/v). The amount of phosphate liberated from ATP was assayed spectroscopically according to Serrano (1978). Precipitated proteins were removed by centrifugation and the entire supernatant was transferred to 940 µl of phosphate reagent [0.5 % (w/v) SDS, 2 % (v/v) H₂SO₄, 0.5 % (w/v) (NH₄)₆Mo₇O₂₄·4 H₂O] and 10 µl of 10 % (w/v) ascorbic acid was added. Absorbance at 750 nm was measured after 10 min at room temperature. Varying phosphate concentrations (0–150 µM) were used as standards to generate a calibration curve. Enzymic activity was calculated from the difference of released PO₄³⁻ between 0 and 120 min. Results were corrected for non-enzymic hydrolysis measured in parallel without membrane preparation. For the control measurements, each sample was measured in the presence of NaVO₃. NaVO₃ (1 mM) was added before the addition of ATP. Results are expressed as the mean \pm SD of triplicate determinations of a representative of three independent experiments.

RESULTS

Conidial swelling and environmental factors triggering germination

Inspection of conidia cultivated submerged in CzD medium revealed the specific stages of conidial germination (Fig. 1). Swelling of dormant conidia occurred during the first 8 h of cultivation, and swollen conidia then turned into germ tubes and continued to grow at a lower rate until a dense ramified mycelial mass was created, which obstructed detailed observations (Fig. 1a). It was apparent from plotting conidia diameter versus time that the diameter changes discontinuously, with a break of the curve at 8 h, the time when the first germ tubes appeared. After this, the biomass production accelerated (Fig. 1b).

To identify factors that determine conidial swelling, the swelling of conidia was observed in a series of media with compositions of increasing complexity. Buffered water and solutions containing NaCl, KCl, and CaCl₂ plus MgSO₄ did not support conidial swelling. Further addition of phosphate dramatically stimulated conidial swelling, which was further increased with the additional presence of nitrate, and with glucose (Table 1). This showed that phosphate is an important factor governing conidial swelling. In addition, yeast autolysate was found to be sufficient to initiate conidial swelling, germ tube formation and hyphal growth (data not shown).

Properties of L-Val uptake

L-Val uptake into germ tubes (after 14 h of cultivation) showed a dramatic increase compared with dormant conidia (non-cultivation) (Fig. 2). Within the first 20 min there

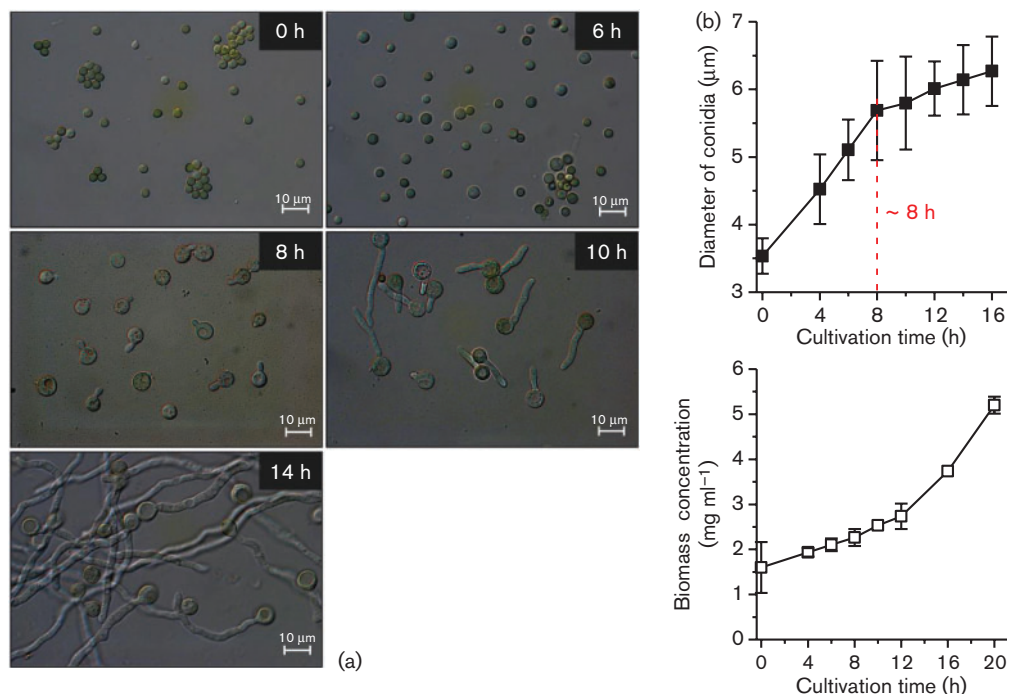


Fig. 1. Germination of *T. atroviride* conidia cultivated submerged in CzD medium under the conditions described in Methods and monitored by differential interference contrast microscopy. Images were made using Axio Imager A1 (Carl Zeiss). Magnification: 100×. (a) Images of conidia in different stages of germination. Bar, 10 μm. (b) Changes in conidial diameters and biomass concentration during germination.

was no difference in L-Val uptake between conidia cultivated in growing (full) medium or non-growing medium (buffered physiological solution). Uptake was moderately stimulated by growing conditions within 1 h (Fig. 2). The following experiments were done under non-growing conditions and uptake was measured for 20 min.

To gain insight into the transport mechanism in dormant and germinated conidia, L-Val transport was measured in the presence of other amino acids (each at 2 mM

concentration). Results of these experiments are summarized in Table 2. L-Val transport was inhibited by hydrophobic and neutral amino acids, such as Ile, Phe, or Ser, Asn, and Gly and, surprisingly, also by L-Lys and L-Arg. Acidic amino acids (Glu, Asp) and β-Ala, sulfur amino acids, Pro, L-Thr and, surprisingly, also L-Leu, stimulated the transport of L-Val. Note that inhibitory and/or stimulatory effects of several amino acids were different between dormant conidia and germ tubes (Table 2).

Table 1. Factors determining swelling and germination of *T. atroviride* conidia

Conidia at 10^7 ml^{-1} were incubated in media of defined composition (all media were buffered with 20 mM MES, pH 6.5) for 20 h at 25 °C, and inspected by light microscopy. Dormant and swollen (after 7 h cultivation) conidia were counted. At least 50 conidia per field were counted, and the mean of four independent counts was calculated. All measurements showed SD of less than 10 %.

Expt. no.	Solute concentration (mM)	Dormant (%)	Swollen (%)
1	Water	100	0
2	50 KCl, 5 NaCl, 10 CaCl ₂ ,	100	0
3	50 KCl, 5 NaCl, 10 CaCl ₂ , 2 MgSO ₄	98	2
4	50 KCl, 5 NaCl, 10 CaCl ₂ , 2 MgSO ₄ , 2 K ₂ HPO ₄	54	46
5	50 KCl, 5 NaCl, 10 CaCl ₂ , 2 MgSO ₄ , 2 K ₂ HPO ₄ , 10 NaNO ₃	45	55
6*	*50 KCl, 5 NaCl, 10 CaCl ₂ , 2 MgSO ₄ , 2 K ₂ HPO ₄ , 10 NaNO ₃ , 20 glucose	35	65

*In this medium, conidia with germ tubes appeared and were considered as swollen conidia.

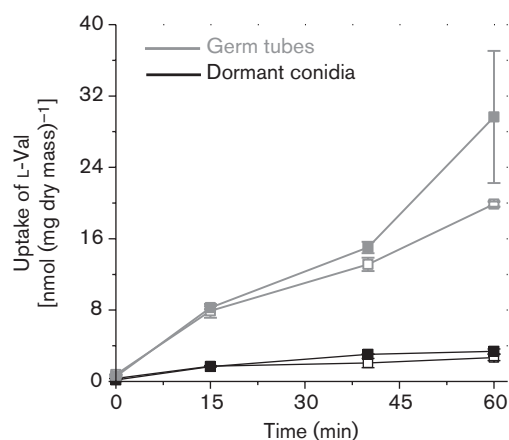


Fig. 2. Kinetics of L-Val transport into dormant conidia (black lines) and germ tubes (after 14 h of cultivation, grey lines) of *T. atroviride* measured under submerged conditions. Before transport experiments, conidia and germ tubes were washed either in CzD medium without YE (conditions enabling growth, filled symbols) or in 1% (w/v) NaCl buffered with 40 mM Tris/HCl, pH 7.4 (non-growing conditions, open symbols). L-Val concentrations were 200 μ M.

Effects of amino acids on L-Val uptake indicated that there must have been some other factor that mediated their inhibitory and/or activating effect besides structural similarity. In the first instance, the PMF was chosen as a possible mediator of the effects of competitor amino acids. Therefore, the effect of TCS on the transport of L-Val was measured.

TCS inhibited L-Val transport in germ tubes at concentrations between 3 and 30 μ M (>80% with 30 μ M) (Fig. 3). The inhibitory efficiency of 30 μ M TCS was, however, much lower when L-Val uptake into dormant or swollen conidia was measured (Fig. 3).

Uptake of polymer precursors during conidia germination

An experiment similar to that shown in Fig. 3 was done also with the transport of URA. This experiment also demonstrated the increase of TCS inhibitory efficiency upon increasing the duration of germination (data not shown). Therefore, experiments were done to monitor the development of the transport of other precursors, in addition to L-Val, namely URA, NAG and CHOL, during germination, and its sensitivity to an uncoupler.

L-Val uptake showed a complex time course during 30–50 h of germination and following growth. Uptake started within 2 h of germination and reached a plateau at 7–12 h. It then decreased dramatically. After 15 h, another step increase of uptake was observed which persisted until the end of the experiment (Fig. 4a). In the presence of an uncoupler (30 μ M TCS) lower uptake was observed.

At time zero, TCS inhibited uptake by 10%, and the extent of inhibition increased up to 85% during the course of measurement.

Uptake of NAG was rapid and a considerable part of this was uncoupler-sensitive from the first 2.5 h of germination. However, after 7.5 h, only an uncoupler-sensitive part of the transport gradually increased and the uncoupler-insensitive part remained unchanged after an increase during the first 2.5 h of this period (Fig. 4b).

URA was transported to a small extent until 10 h after which its uptake gradually increased until 26 h, and then decreased. This transport was TCS-sensitive, and the TCS-insensitive transport increased in the later phase of germination, after 10–15 h (Fig. 4c). The two components of URA uptake appear to change in a coordinated manner.

Uptake of CHOL was different from that of URA in the early phase (up to 8 h), when it increased to by 160% and later transiently increased up to 600%, and then decreased similarly as with transport of URA. These changes were almost completely TCS-sensitive. The TCS-insensitive part increased slowly during cultivation (Fig. 4d).

Although the time courses of the uptake of these metabolites were similar, specific uptakes were substantially different: 10 nmol (mg dry mass)⁻¹ h⁻¹ for L-Val but only 0.1 nmol (mg dry mass)⁻¹ h⁻¹ for URA and NAG, and 1 nmol (mg dry mass)⁻¹ h⁻¹ for CHOL.

Table 2. The effect of amino acids on L-Val transport into *T. atroviride* conidia

Amino acids tested	Transport (% of control)	
	Dormant conidia	Germ tubes (after 14 h cultivation)
Control	100	100
L-Gly	89	122
L-Asp	224	576
L-Glu	274	329
L-Asn	84	302
β -Ala	114	225
L-Met	121	44
L-Arg	91	95
L-Ser	84	20
L-Cys	133	52
L-Pro	179	135
L/D-Thr	160	128
L-Leu	193	138
L/D-Lys	12	64
L-Ile	13	83
L/D-Phe	52	63

Conidial suspension was pre-incubated with 2 mM of the respective amino acid for 5 min and then the radioactively labelled L-Val (200 μ M) was added. Transport was measured under non-growing conditions as described in Methods.

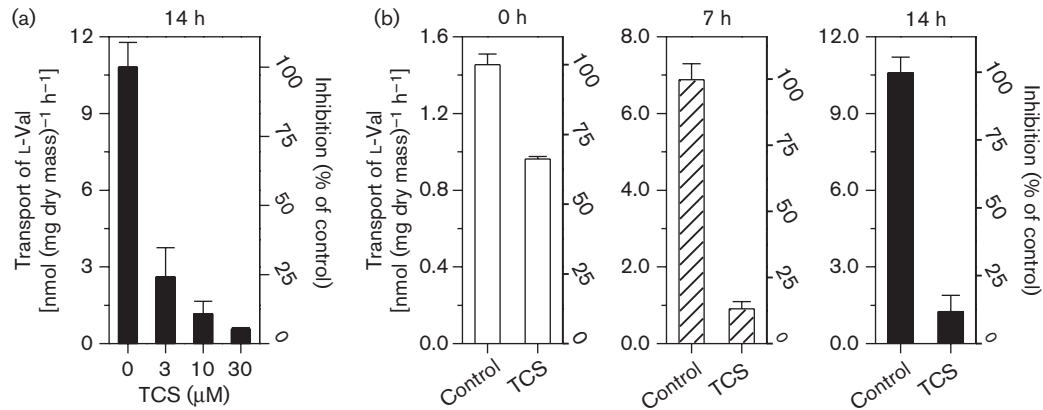


Fig. 3. The effect of TCS on the transport of L-Val into conidia germinated for different times. (a) The concentration dependence of the effect of TCS on L-Val transport into germ tubes. (b) The efficiency of 30 μ M TCS on dormant conidia (0 h), swollen conidia (after 7 h of cultivation) and germ tubes (after 14 h of cultivation). Conditions of transport measurement were: non-growing conditions, pH 7.4, L-Val concentration 200 μ M. The same volume of methanol (1 %, v/v) was present in the control. Note differences in the y-axis scales.

Substrate analogues of URA, such as uridine, thymidine or the purine bases hypoxanthine or 6-mercaptopurine were tested for their effect on URA transport (Fig. 5c). Only 6-mercaptopurine inhibited the transport of URA. Similarly, substrate analogues of NAG, such as glucosamine, or hexoses (glucose, fructose) but not galactose exerted some inhibitory effect on the transport of NAG (Fig. 5b).

ATPase inhibitors and activity of H⁺-ATPase and *pma1* gene expression in Δ *pma1* strains

In addition to TCS, the uptake of all precursors was inhibited by azide, and the transport of NAG and URA was inhibited by vanadate, compounds that are known to inhibit transport of ATPases (Fig. 5a–c). Azide consistently inhibited transport of all nutrients unlike vanadate, which failed to inhibit transport of L-Val and CHOL. These inconsistencies could be ascribed to the multiplicity of vanadate actions, known also as a protein phosphatase inhibitor (Gordon, 1991).

The effects of uncoupler, vanadate and azide led us to propose that a PMF is necessary for the uptake of macromolecule (and lipid) precursors (Figs 3–5) and that this PMF is generated by the plasma membrane H⁺-ATPase. Therefore, the activity of this enzyme and the expression of its gene were measured in germinating conidia. These experiments showed that activity of H⁺-ATPase was virtually zero in dormant conidia and in conidia germinated for less than 9 h (Fig. 6a). This corresponds well with the time course of *pma1* gene expression (Fig. 6b). To corroborate the role of the H⁺-pump in the transport of macromolecule precursors within conidia germination, the uptake of L-Val into the Δ *pma1* mutant strain of *T. atroviride* was studied. The Δ *pma1* strain was very sensitive to shaking in submerged culture [CzD medium

with 0.5 % (w/v) YE]. In addition, the mutant strain completely lost the ability to form conidia on solid CzD medium (Olejníková *et al.*, 2011). Measurements of L-Val uptake into aerial mycelium of both parental and mutant strains showed that the Δ *pma1* strain has a considerably lower rate of L-Val uptake [0.10 ± 0.01 nmol (mg dry mass)⁻¹ h⁻¹] than that of the WT strain [0.22 ± 0.04 nmol (mg dry mass)⁻¹ h⁻¹] and the degree of inhibition of L-Val uptake by TCS was 70 % in the mutant and 89 % in the WT (Fig. 4e).

DISCUSSION

Transport is an integral part of metabolism. This rule may be strictly correct for growing cells but may not be so for dormant forms of micro-organisms, such as fungal conidia. Conidia may break dormancy and swell even in demineralized water (Schmit & Brody, 1976), and the availability of external carbon and nitrogen compounds is required during later phases of germination. *T. atroviride* conidia do not swell either in water or in media containing simple salts, such as KCl, NaCl and MgSO₄, but the addition of phosphate salts seems to be sufficient for conidial swelling, which could be further stimulated by the addition of nitrogen and carbon sources (Table 1). Dormant conidia took up L-Val under non-growing conditions, indicating that a functional transport system(s) was present. These transport systems may represent a part of the machinery signalling whether extracellular conditions are suitable for germination. Such explanation, although feasible, needs to be supported by additional experiments. In light of the recent data by Hayer *et al.* (2013, 2014), there is only a loose relationship between the transport of nutrients and support for germination and/or growth. Conditions enabling growth slightly

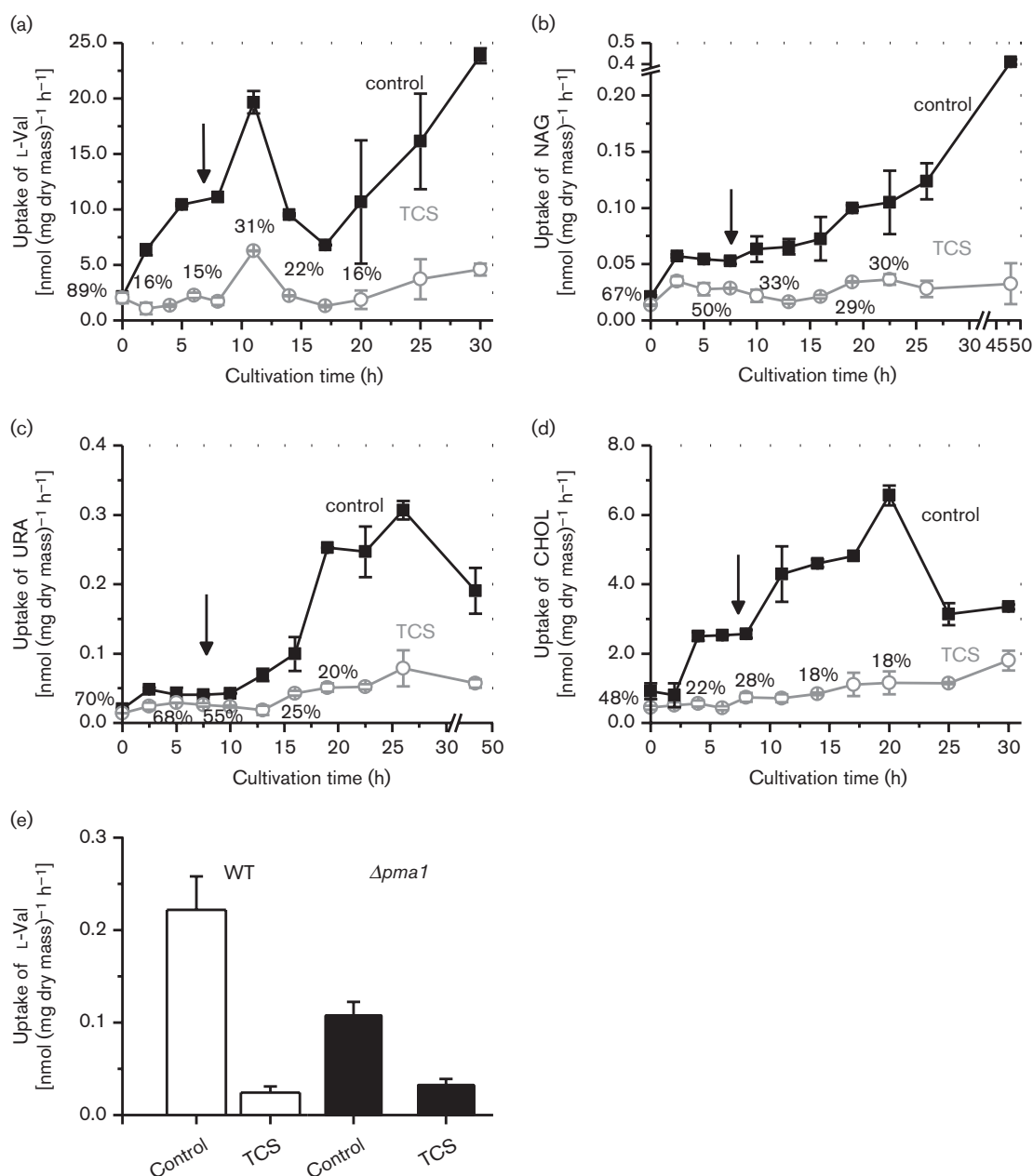


Fig. 4. Development of TCS-sensitive and TCS-insensitive uptake of L-Val (a), NAG (b), URA (c) and CHOL (d) during the germination of conidia under submerged conditions. Transport of precursors was measured under non-growing conditions. (e) The effect of TCS (30 μ M) on uptake of L-Val into the aerial mycelium of the WT and $\Delta pma1$ mutant strains of *T. atroviride*. TCS inhibition of nutrient uptake is expressed as a percentage of the control measurement. Arrows indicate the end of the initial swelling of conidia.

enhanced the uptake in both dormant conidia and in those germinated up to the phase of germ tube formation, within 1 h of transport measurement (Fig. 2). This probably reflects higher amino acid requirements under conditions enabling growth.

The increase of L-Val uptake into germ tubes was almost an order of magnitude higher than that of dormant conidia (Fig. 2). As the PMF is known to be involved in fungal

amino acid transport (Kropf *et al.*, 1984; Eddy & Hopkins, 1988; Olivera *et al.*, 1993; Pokorný *et al.*, 2004), the effect of uncoupler was tested. The degree of inhibition increased from about 10–30 % in dormant conidia to 80–90 % in germinated conidia, suggesting an increased requirement of PMF by amino acid permeases during germination. This could be due to the involvement of different amino acid permeases during the early and later phases of

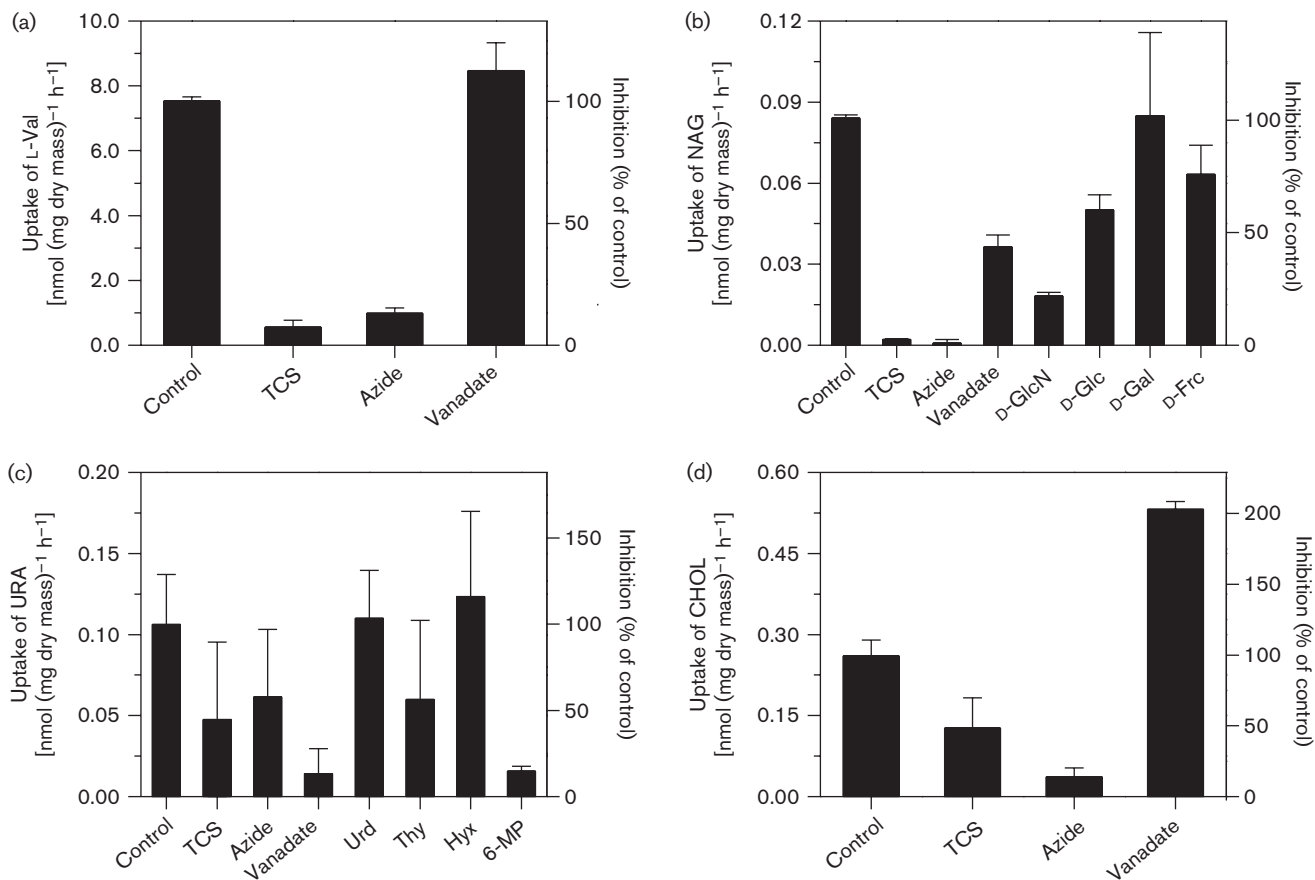


Fig. 5. The effect of inhibitors and substrate analogues on the uptake of L-Val (a), NAG (b), URA (c) and CHOL (d) into *T. atroviride* conidia cultivated under submerged conditions. TCS concentration was 30 μM , while other compounds were tested at a concentration of 2 mM. The concentration of L-Val, NAG, URA and CHOL was 200 μM . Conidia were germinated for 7 h and used for transport measurements under non-growing conditions. D-GlcN, *N*-acetyl-D-glucosamine; D-Glc, D-glucose; D-Gal, D-galactose; D-Frc, D-fructose; Urd, uridine; Thy, thymine; Hyx, hypoxanthine; 6-MP, 6-mercaptopurine.

germination. The competition of non-labelled amino acids with the ^{14}C -Val transport shown in Table 2 could be considered as evidence for the involvement of multiple amino acid permeases in the course of germination, in agreement with the data of Tisdale and DeBusk (1970), Railey & Kinsey (1976) and Tazebay et al. (1995, 1997). Another explanation for the difference in uncoupler effect could be the development of PMF during this period. This does not, however, exclude the first explanation presented above.

If the PMF has some developmental role in germination, it should influence not only amino acid transport but also the transport of other solutes. This was well documented by the experiments with precursors of other macromolecules (Figs 4 and 5). There are, however, differences between the uptakes of these nutrients. Uptake of L-Val and NAG increased up to the end of the experiment (30 and 50 h, respectively), whilst uptake of URA and CHOL reached a plateau after about 20 h. We suggest that the net URA uptake is attenuated once rRNAs and mRNAs

are present in sufficient numbers and/or pyrimidine biosynthesis is established. The same reasoning could be applied to explain the attenuation of CHOL uptake.

Uptake of URA contained a large TCS-insensitive part. This is in agreement with our previous observation (Lakoš et al., 1999) that URA is transported by two transport systems with different affinities. Only the high-affinity transport was strongly dependent on the PMF. Under the conditions applied here, we measured the activities of both transport systems. Measurements of both purine transport and of expression of purine transporter genes during germination of *A. nidulans* conidia also revealed that multiple transport proteins are involved in purine transport during germination, which are under multiple control sites (Amillis et al., 2004; Vlanti & Diallinas, 2008).

Similar reasoning could be applied to explain of both the lag in and the attenuation of CHOL uptake. Conidia contain both choline and betain and their concentration increases during germination (Kaliňák et al., 2014). The

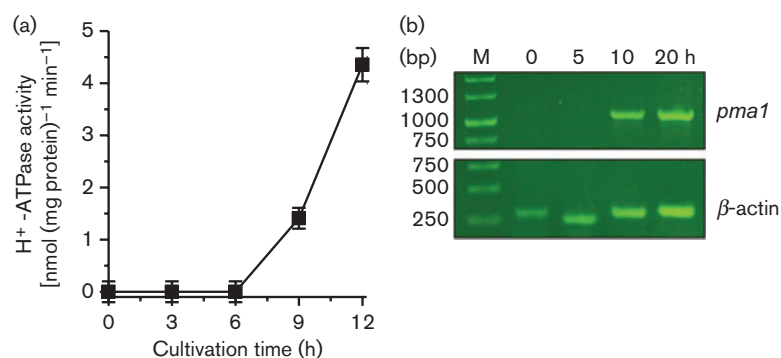


Fig. 6. H⁺-ATPase activity (a) and expression of the *pma1* (H⁺-ATPase) gene (b) during *T. atroviride* conidia germination. (a) Activity of H⁺-ATPase was measured in microsomal fractions of homogenates of conidia germinated for the time indicated according to the procedure described in Methods. (b) Expression of H⁺-ATPase was measured from cDNA prepared from conidia germinated for the time indicated.

synthesis of fatty acids, which precedes synthesis of phospholipids, is downregulated at the early stage of germination (Novodvorska *et al.*, 2013). This may indicate that choline taken up by conidia is converted to betain rather than to phospholipid biosynthesis during the early phase of germination, as choline oxidase has low activity in non-germinated conidia (*Aspergillus fumigatus*) and its activity increases during germination (Lambou *et al.*, 2013). Its transcript is present also in mycelia of *Trichoderma reesei* (Metz *et al.*, 2011). NAG uptake may be delayed for similar reasons, as NAG is required for the synthesis of chitin during development of germ tubes and mycelia.

The common features to transport of all the measured nutrients may result from the increased generation of PMF. This is supported by the activity of H⁺-ATPase and of expression of its gene. These two parameters appear between 6–9 h and 5–10 h of germination, respectively (Fig. 6), i.e. approximately at the time of symmetry breaking (Fig. 1). Thus, it is feasible that the generation of PMF followed by enhanced nutrient fluxes accelerates conidial metabolism, conveying it to the phase of hyphal growth. Unfortunately, it is not possible to prove this by means of genetic experiments with the disrupted *pma1* gene. We have previously isolated such mutants (Olejníková *et al.*, 2011) and found they do not form conidia. Neither mutation of the *pma1* gene nor the effect of TCS on L-Val uptake resulted in complete loss of uptake of L-Val into surface mycelium of *T. atroviride*. This indicates the existence of an alternative system (besides H⁺-ATPase activity), which may generate a PMF across the plasma membrane and drive the influx of L-Val.

The present experiments also revealed that uncoupler sensitivity uptake of all macromolecule precursors takes place before H⁺-ATPase is expressed. This raises the question of the source of the PMF in dormant conidia or during the early phase of germination, and in the $\Delta pma1$ mutant.

This putative PMF source remains obscure, but there may be clues towards future research. First, the PMF may be generated by the activation of an Na⁺/H⁺ antiporter, which can use the sodium gradient to pump out H⁺ from the cytoplasm. Such a transport system has been identified in *A. nidulans* (Kamauchi *et al.*, 2002). The observations of Slayman & Slayman (1968) and Eddy & Nowacki (1971) who observed the exchange of K⁺ for both Na⁺ and H⁺ in *N. crassa* and yeasts, respectively, do not seem to support this hypothesis. Second, it is feasible that germinating conidia produce organic acids, which could be transported by anion–proton symport, driven by glycolysis and the TCA cycle, major producers of cytoplasmic H⁺. Such a transport system has been considered in red blood cells and in Ehrlich ascites cells (Johnson *et al.*, 1980), in bacteria (reviewed by Konings *et al.*, 1992) and in fungi, which are known to be powerful producers of organic acids (reviewed by Konings *et al.*, 1992; Burgstaller, 1997, 2006). Support for this explanation of the uncoupler sensitivity should be obtained by future direct measurements of membrane potential, fluxes of organic acids, and Na⁺ and H⁺.

In summary, the results here show that expression of the H⁺-ATPase gene and H⁺-ATPase activity starts after breaking symmetry in conidial germination. This is accompanied by stimulation of the uptake of selected precursors of biopolymers and phospholipids, suggesting that the H⁺-ATPase activity is an accelerator of hyphal development in the later phase of germination.

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