Root exudates stimulate the uptake and metabolism of organic carbon in germinating spores of *Glomus intraradices*

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Summary

• Root exudates play a key role during the presymbiotic growth phase and have been shown to stimulate hyphal branching and the catabolic metabolism of arbuscular mycorrhizal (AM) fungal spores.

• Here, the effect of root exudates on presymbiotic growth, uptake of exogenous carbon and transcript levels for genes putatively involved in the carbon metabolism of germinating spores were determined.

• Crude root exudates led to a slight acceleration of spore germination, increased germ tube branching and stimulated uptake and catabolic metabolism of acetate, and to a greater extent of glucose, but had no effect on gene expression. By contrast, partially purified root exudates increased the transcript levels of acyl-CoA dehydrogenase (ß-oxidation of fatty acids to acetyl-CoA), malate synthase (glyoxylate cycle) and glutamine-fructose-6-phosphate aminotransferase (chitin biosynthesis), but did not differ from crude root exudates in their effect on substrate uptake and respiration. The expression of glycogen synthase (glycogen biosynthesis), glucose-6-phosphate dehydrogenase (pentose phosphate pathway) and neutral trehalase (hydrolysis of trehalose) were only marginally or not affected by root exudates.

• Root exudates have an effect on both membrane activity and gene expression and the results are discussed in relation to the catabolic and anabolic metabolism of spores during presymbiotic growth.

Key words: acyl-CoA dehydrogenase, arbuscular mycorrhiza (AM), carbon metabolism, glutamine-fructose-6-phosphate aminotransferase, malate synthase, presymbiotic growth, respiration, root exudates.

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Introduction

Arbuscular mycorrhizal (AM) fungi form mutualistic associations with the roots of > 80% of all known land plant species (Smith & Read, 1997). The mycorrhizal fungus explores the soil extensively with its extraradical mycelium and contributes, especially under conditions of low external supply, significantly to the nutrient uptake of the host plant. In exchange for this beneficial effect on nutrient uptake, the host plant transfers up to 20% of its photosynthetically fixed carbon to the fungal symbiont (Jakobsen & Rosendahl, 1990; Wright *et al.*, 1998). The AM fungi are asexual obligate symbionts that are completely dependent on carbon supplied by their host plant's roots and are incapable of completing their life cycle without them.

Arbuscular mycorrhizal fungal spores are able to germinate asymbiotically, but the presymbiotic growth of the spores is highly dependent on root exudates and it has been suggested that plants that are already colonized by AM fungi control

their further colonization by the quantity and quality of their root exudates (Pinior *et al.*, 1999; Gadkar *et al.*, 2003). Root exudates have been shown to stimulate hyphal branching (Bécard & Piché, 1989; Buée *et al.*, 2000; Nagahashi & Douds, 2000), the uptake of phosphate by germ tubes (Lei *et al.*, 1991) and the hyperpolarization of the fungal plasma membrane (Ayling *et al.*, 2000; Ramos *et al.*, 2008). In addition to their effect on anabolic processes, root exudates stimulate the catabolic metabolism of germinating spores by an increase in mitochondrial biogenesis and the upregulation of genes related to mitochondrial activity (Tamasloukht *et al.*, 2003; Besserer *et al.*, 2006).

Plant roots release a variety of different compounds such as sugars and sugar alcohols, amino acids, fatty acids, phenolics, organic acids, phytosiderophores, vitamins, plant growth regulators, purines, nucleosides, proteins and peptides, polyamines, inorganic ions and volatile molecules such as CO_2 and H_2 into the rhizosphere (Dakora & Phillips, 2002). Whether a single compound or multiple plant signals trigger the different responses of spores in the presymbiotic growth phase, is still unknown (Jones *et al.*, 2004); however, the wide range of compounds reported to affect presymbiotic hyphal growth, branching and colonization efficiency favor the latter possibility. These compounds include flavonoids (Gianinazzi-Pearson *et al.*, 1989, Scervino *et al.*, 2006), volatiles (Bécard *et al.*, 1992; Balaji *et al.*, 1995), mannitol (Kuwada *et al.*, 2005) or hydrophobic compounds (Tarawaya *et al.*, 1998). Recently, Akiyama and coworkers (Akiyama *et al.*, 2005; Akiyama & Hayashi, 2006) isolated a branching factor from the root exudates of *Lotus japonicus* and identified it as strigolactone. Strigolactones have been shown to stimulate the cell proliferation and the mitochondrial density in the hyphae of the AM fungus *Gigaspora rosea* (Besserer *et al.*, 2006), but also the seed germination of the parasitic weeds *Striga* and *Orobanche* (Matusova *et al.*, 2005).

Our present knowledge about uptake and metabolic processes that are involved in the development of the AM symbiosis during the presymbiotic growth phase is still limited. The main goal of this study was to analyse the effect of root exudates on uptake and metabolism of exogenous carbon sources and on fungal gene expression to get more information on how root exudates affect the presymbiotic growth of germinating spores. A better understanding of these processes could help to elucidate reasons for the obligate biotrophy of AM fungi and could also contribute to new strategies in the application of these fungi in sustainable agriculture.

Materials and Methods

Spore material

For the experiments spores of *Glomus intraradices* Schenck & Smith (DAOM 181602) provided by Premier Tech Biotechnologies (Mycorise ASP, Rivière-du-Loup, Canada) were used. The spores were kept refrigerated at 4°C. Before the start of the experiments the spores were blended at high speed for 45 s in a commercial blender, filtered and transferred into autoclaved H_2O. The spore suspension was continuously stirred and a defined volume with a homogenous number of spores was added to the Petri dishes for the experiments.

Root exudate synthesis and purification

For the synthesis of root exudates transformed nonmycorrhizal roots of *Daucus carota* L. (DC-I clone) were first cultured in liquid mineral medium (St. Arnaud *et al.*, 1996) containing 3% sucrose. After 4 wk the roots were carefully removed, washed twice with sterile water and then transferred into a flask containing 1 L of sterile water. The roots were cultured on a shaker at room temperature conditions and after 1 wk the solution containing the root exudates was harvested. The root exudate was stored for further use at −20°C. In addition, crude root exudates were partially purified and concentrated by using a C18 SEPAK cartridge (1000 mg tube, octadecylsilyl; Lida Manufacturing, Kenosha, WI, USA). After loading the cartridge with 100 ml of the crude exudate, the cartridge was first washed with 2 ml 35% acetonitrile. After this wash step the partially purified root exudates were collected after loading the cartridge with 3 ml 70% acetonitrile followed by 1 ml 100% acetonitrile, dried under nitrogen and dissolved in 0.5 ml 70% methanol (200 fold concentrated). The data for these partially purified root exudates were compared with a control to which 70% methanol was added (the final methanol concentration in the medium was 5.4%). The glucose concentration of the root exudates was analysed by gas chromatography–mass spectrometry (GC-MS) (Pfeffer *et al.*, 2004) and was 34 μg l^{-1} or 4.6 μg l^{-1} for crude or partially purified root exudates, respectively.

Experimental design

Germination and growth The activity of the root exudates was tested by a germination and branching assay. Spores of *G. intraradices* were transferred onto solid medium (no sucrose; St. Arnaud *et al.*, 1996) containing 7.7% (v : v) of the crude root exudates or 2.4% (v : v) of the partially purified root exudates. Approximately 50–70 spores were added onto the medium and the germination and hyphal branching was counted between 1 d and 16 d in 1–2 d intervals using a stereomicroscope.

Substrate uptake Aliquots of 500 μl of the spore suspension with approx. 4000 spores was added to Petri dishes containing liquid mineral medium (no sucrose; St Arnaud *et al.*, 1996) enriched with 1 μM, 2.5 μM, 5 μM, 10 μM, 25 μM or 50 μM [1-^{14}C]acetate (specific activity 39.0 mCi $mmol^{-1}$) or [U-^{14}C] glucose (specific activity 317 mCi $mmol^{-1}$). After 1 d, 2 d, 4 d, 6 d and 8 d aliquots of the medium were taken and the residue of the initially added ^{14}C concentration was determined by liquid scintillation counting ($n = 8$). After 8 d, > 95% of the originally added substrates were taken up and a second pulse

with the same concentration was added to the spores. The medium was analysed for further 1, 2, 4 and 6 d. After 2 wk at 30°C (8 d first pulse and 6 d second pulse) the spores were collected and prepared for liquid scintillation counting.

To examine the effect of root exudates on substrate uptake by germinating spores, 500 µl of the spore suspension with approx. 4000 spores was added to Petri dishes containing liquid mineral medium (no sucrose; St Arnaud *et al.*, 1996), enriched with 10 µM or 50 µM [1-^{14}C]acetate or [U-^{14}C]glucose and 7.7% of the crude or 2.4% of the partially purified root exudates (v : v). After 4 h and 8 h, 1 d, 2 d and 4 d at 30°C aliquots of the medium were taken and analysed by liquid scintillation counting. After 4 d the spores were collected and prepared for liquid scintillation counting. Respiration was calculated by the subtraction of the sum of the ^{14}C content of the residue and the fungal biomass from the initially added ^{14}C content.

^{13}C labeling Spores were cultured in mineral medium (9 ml, no sucrose; St. Arnaud *et al.*, 1996) containing 4 mM [1-^{13}C]acetate or 25 mM [1-^{13}C]glucose and 7.7% of crude root exudates. After 8 d the spores were harvested, lyophilized, weighed, extracted and prepared for nuclear magnetic resonance (NMR) investigation (see later).

Gene expression The expression of genes putatively involved in the carbon metabolism of germinating spores (Bago *et al.*, 1999, 2000) was tested by adding spores to Petri dishes containing mineral medium enriched with 7.7% crude root exudates or 2.4% (v : v) partially purified root exudates. The spores were harvested by filtration after 2 h, 4 h, 8 h, 24 h, 48 h and 96 h incubation at 30°C and stored at −80°C. In addition, the protein levels of isocitrate lyase (ICL) depending on the root exudate concentration (0.7% and 7.7% of crude root exudates and 2.4% of partially purified root exudates) was examined after incubating spores in mineral medium enriched with 0 or 50 µM acetate. After 24 h the spores were collected and stored at −80°C and prepared for Western blots.

Liquid scintillation counting

The samples were dried in an oven at 70°C, weighed and digested with a tissue solubilizer (TS-2, RPI Corp., Mount Prospect, IL, USA) or measured directly without solubilization (medium samples). After complete digestion the samples were suspended in scintillation cocktail (Biosafe II; RPI Corp., Mount Prospect, IL, USA) and the radioactivity was determined by liquid scintillation counting (LS 6500; Beckman Coulter, Fullerton, CA, USA) with a correction of the counting accuracy by use of an internal standard.

Nuclear magnetic resonance

After lyophilization, the samples were weighed and the carbohydrates were extracted with acid washed sand in 70% methanol by grinding the tissue in small tubes with a motor driven pestle. After filtration the methanol was evaporated under nitrogen and the aqueous solution was freeze-dried. For NMR the extracts were dissolved in 750 µl deuterated water and insoluble matter was removed by centrifugation. ^{13}C-isotopic abundance (atom per cent ^{13}C) of the labeled positions of a given compound were calculated by comparison with ^{13}C-isotopic signals of unlabeled positions of the same compound and/or by measurement of the ^{13}C-^{1}H satellites of ^{1}H signals in proton spectra. Conditions and instrumentation for obtaining ^{1}H and ^{13}C NMR spectra were as previously described (Bago *et al.*, 1999; Pfeffer *et al.*, 1999, 2004) using a 400 MHz instrument (Varian, Palo Alto, CA, USA) for ^{13}C and a 750 MHZ instrument (Bruker, Billerica, MA, USA) for ^{1}H spectroscopy.

RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

The total RNA from the spores was extracted by using the RNeasy mini RNA isolation kit of Qiagen (Valencia, CA, USA). The RNA samples were treated with DNase I (Ambion, Austin, TX, USA) and quantified by the RiboGreen fluorescence assay of Molecular Probes (Eugene, OR, USA). Reverse transcription and polymerase chain reaction (PCR) were performed in duplicate with 1 ng of total RNA from each of the three independent biological replicates. The thermal cycling conditions were as follows: 60 min at 48°C for reverse transcription, 10 min at 95°C to denature, and 45 cycles of 15 s at 95°C and 1 min at 60°C. A no-RT control reaction was included for every primer set which resulted in Ct values of > 45, proving the effectiveness of the DNase I removal of genomic DNA. Gene-specific PCR products were monitored in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using TaqMan probes for optimal specificity. The changes in expression of the selected genes were determined by calculating the difference in the critical threshold (ΔCT) values of the PCR cycle at which the change in fluorescence becomes exponential. Fold-change calculations were made by using the comparative ΔΔCT method where fold induction = $2^{-\Delta\Delta CT}$ (Pfaffl, 2001). The term ΔΔCT is defined as

$$[CT_{GI}\ (\text{exudate treatment}) - CT_{RG}\ (\text{exudate treatment})] - [CT_{GI}\ (\text{control}) - CT_{RG}\ (\text{control})]$$

(GI indicates the gene of interest and RG indicates the reference gene). The efficiency of the PCR reaction was tested by measuring the CT values for each GI/RG pair in dilution series (Applied Biosystems, 2001). The slope of the resulting line was 0.19 or less.

For the normalization of the gene expression data an expressed sequence tag (EST) with a sequence similarity to a fungal siderophore regulatory protein (SRP) and the S4 ribosomal

Table 1 Forward and reverse primers and dual-labeled fluorogenic probes used for the gene expression studies

Gene		Sequence
Acyl-CoA dehydrogenase (ACD)	Forward	5′-GATGTTATTCGTAATAAACTTGCCCATA-3′
	Reverse	5′-TGTTTGATAAATTAATGACTCCATCCA-3′
	Probe	5′-CGCGTAAAATTGAGGCAACCCATGC-3′
Glucose-6-phosphate dehydrogenase (G6PDH)	Forward	5′-TCCTCAACCTTACGCATACGG-3′
	Reverse	5′-CGGCCTCTTGAATCCATATTTAGA-3′
	Probe	5′-CAAACTCTTCAATACCAGGTGGACCACGTG-3′
Glycogen synthase (GLYS)	Forward	5′-AACAGCTTGACCTTTCAGTGCTT-3′
	Reverse	5′-CCGTTGTTGCATTTATTGTCATG-3′
	Probe	5′-CACAGCAAATGATTGAGTTGCCGCA-3′
Malate synthase (MS)	Forward	5′-TTGGTTGTGTACCAATCCATAATCTT-3′
	Reverse	5′-CCATTGCCATAATTGTGAACGT-3′
	Probe	5′-TTTCTGCTGTTGCCGCATCTTCCA-3′
Trehalase (TE)	Forward	5′-TTTCAAGTTTGTGCCTCGTGA-3′
	Reverse	5′-GCCCTCTCCATATGATGTGTAAGATA-3′
	Probe	5′-ACTTGATATGAAGCGTTCATCCAACCGAAA-3′
Glutamine-fructose-6-phosphate aminotransferase (GFAT)	Forward	5′-GGGCTACTCACGGTGAACCTT-3′,
	Reverse	5′-CACAGTAAATTCGCCCTTTGG-3′
	Probe	5′-TCCAACGAATTCACATCCTCATCGTTCTG-3′
S4 ribosomal protein (RP)	Forward	5′-TCTTGTGAAGGTTGATGGCAAA-3′
	Reverse	5′-CGCCATTTCTTTCGATCGA-3′
	Probe	5′-TTCGAACCGATTCAACATACCCTGCC-3′
Siderophore regulatory protein (SRP)	Forward	5′-CTTTATCTCAAGGCTCGAAACACA-3′
	Reverse	5′-CAGGAGGAGCTTTCTTAATTGCAT-3′
	Probe	5′-TCGTCCGCCATGGTTGAAACGC-3′

protein of *G. intraradices* were used. Since the CT values of the S4 ribosomal protein indicated a slight induction of this gene in response to crude root exudates, but not in response to partially purified root exudates, SRP was used to normalize the gene expression data shown in Fig. 4. However, the overall effects of root exudates on gene expression of germinating spores did not differ significantly between the two reference genes (data not shown). The following genes were analysed: acyl-CoA dehydrogenase (ß-oxidation of fatty acids to acetyl-CoA, ACD, AY033936); glucose-6-phosphate dehydrogenase (oxidative pentose phosphate pathway, G6PDH, EU478725); glycogen synthase (biosynthesis of glycogen from glucose via UDP-glucose, GLYS, BE603748); malate synthase (glyoxylate cycle, MS, BE603747); neutral trehalase (breakdown of trehalose to its glucose subunits, TE, B1246186); and glutamine-fructose-6-phosphate aminotransferase (biosynthesis of chitin, GFAT, BE603749) (Table 1). A minimum of a twofold change in expression in all biological replicates was considered to be biologically relevant. The data represent the mean and SEM of three biological replicates ($n = 3$).

Western blots

To determine the protein levels of isocitrate lyase (glyoxylate cycle, ICL, EC 4.1.3.1) in spores after root exudate incubation, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting were carried out. The fungal proteins were extracted in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (NaOH, 50 mM, pH 7.8) containing 100 mM NaCl, 0.05% SDS, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), 1 mM aminocaproic acid, 1 mM benzimidine, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT) by using a small tube and a motor-driven pestle followed by a sonification step (Sonifier 450; Branson, Danbury, CT, USA). The protein yields were determined with the Bradford protein assay (Sigma, St Louis, MO, USA) and were 0.37 ± 0.02 μg mg^{-1} FW for the controls (before the treatment) and 0.70 ± 0.07 μg mg^{-1} FW or 1.03 ± 0.12 μg mg^{-1} FW with or without acetate addition, respectively. A SDS-PAGE was performed to resolve the proteins after loading 3.5 μg proteins onto the lanes. The concentration of the stacking gel and separating gel was 4% and 10%, respectively. The proteins were transferred to Hybond ECL (PVDF) membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were incubated in a blocking solution containing 5% milk powder in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.3% Tween (TBST) buffer and then incubated overnight with the primary antibodies in TBST. The specific antibodies were designed against the *N*- and *C*-terminus of ICL and were purified from the serum by Anaspec. (San Jose, CA, USA). The antibodies reacted sensitively with the ICL band at

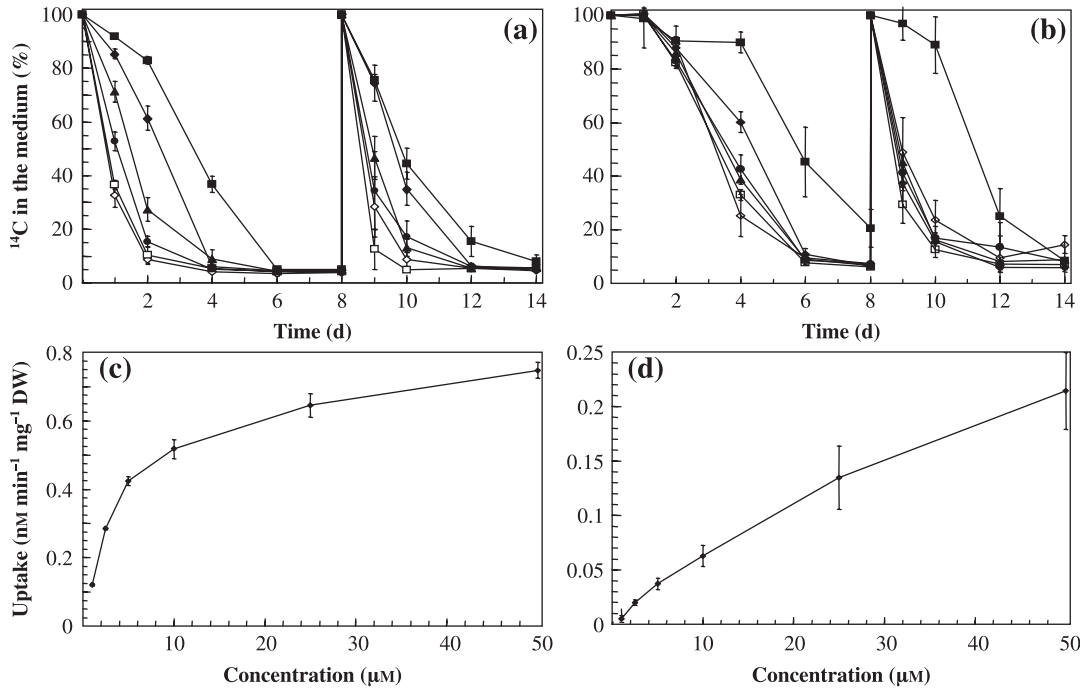

Fig. 1 Uptake of acetate (a,c) and glucose (b,d) by germinating spores of *Glomus intraradices*. (a and b) Uptake is given as the percentage of the residue from the initially added ^{14}C concentration. The substrates were added twice to the spores, at the start of the experiment and after 8 d. (c and d) Uptake calculated as nM min^{-1} mg^{-1} DW of the germinating spores. Means (n = 8) ± SEM (vertical bars) are shown. Symbols in (a and b): 1 μM (open diamonds), 2.5 μM (open squares), 5 μM (closed circles), 10 μM (closed triangles), 25 μM (closed diamonds), 50 μM (closed squares).

approx. 60 kDa in extracts of spores, but showed no reactions with protein extracts of uncolonized or colonized roots of *D. carota* (not shown). After incubation in the secondary antibody in TBST (rabbit IgB, horseradish peroxidase linked), the blots were developed using an enhanced chemiluminescence detection kit (ECL; Amersham Biosciences) or stained using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT).

Statistical treatment

Mean values and vertical bars representing standard errors are shown in the Figures. The results are stated as significant if a statistically significant difference ($P \leq 0.05$) between the treatments by ANOVA and Fisher's LSD test (Unistat Software, London, UK) was found. Significance is indicated in figures by different letters.

Results

The effect of root exudates on presymbiotic growth

The presence of root exudates was not required for spore germination of *G. intraradices*, but crude root exudates slightly accelerated the germination of the spores and stimulated the branching of the germ tubes (not shown). After 8 d, germinating spores had 8.9 ± 1.8 branches under control conditions and 14.1 ± 1.2 branches after the addition of crude root exudates. The stimulation of branching by crude root exudates had no significant effect on the biomass (not shown), since the number of branches but not the length of the branches was affected. When partially purified root exudates were added, the spores germinated later and the number of branches after 14 d did not differ from the control spores.

The uptake of exogenous organic carbon by germinating spores

Both, acetate and glucose were taken up by germinating spores of *G. intraradices* (Fig. 1a,b). The uptake of acetate was first detected 4 h after acetate addition (Fig. 1a), which was before visible spore germination. The acetate uptake followed Michaelis–Menten kinetics with an apparent V_{max} of 0.90 nM mg^{-1} DW min^{-1} and a K_m of 6.3 μM (Fig. 1c). By contrast a lag phase of approx. 24 h was observed for the uptake of glucose and the uptake followed a more linear dependence on concentration within the range used (1–50 μM; Fig. 1b,d). The rate of acetate uptake was considerably higher than the uptake rate for glucose. When germinating spores were supplied with 10 μM of either substrate, 72.7 ± 4.9% of the acetate but only 14.0 ± 2.1% of the

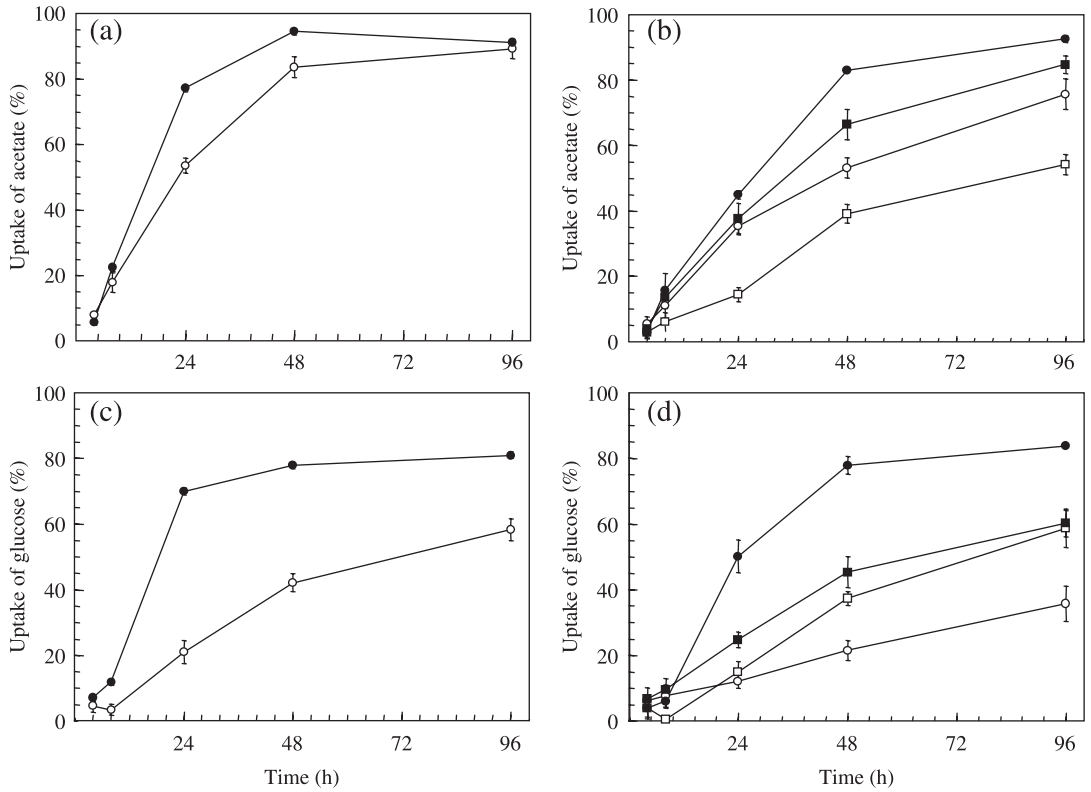

Fig. 2 Effects of root exudates on the uptake of 10 μM (a, c) or 50 μM (b,d) acetate (a,b) or glucose (c,d) by germinating spores of *Glomus intraradices*. Means (n = 8) ± SEM (vertical bars) are shown. Symbols: control water (open circles), control + methanol (open squares), 7.7% root exudates (closed circles), partially purified root exudates (closed squares).

glucose was taken up within 2 d (Fig. 1a,b). However, the uptake of glucose was faster when glucose was resupplied to the spores 8 d after the first addition. After the second addition the rate of glucose uptake did not differ significantly from the uptake of acetate when both were present at 10 μM. One day after the resupply 46.6 ± 8.1% and 45.1 ± 3.8% of acetate and glucose, respectively, had been taken up by the spores.

The effect of root exudates on carbon uptake and utilization

The uptake of both acetate and glucose was significantly stimulated by the presence of root exudates in the medium (Fig. 2). This increase was not primarily caused by a stimulation of germination or hyphal branching, since the stimulation of uptake could be observed 8 h after transfer of the spores into the solution, which was before the germination of most of the spores. Both crude and partially purified root exudates had a similar effect when compared with their specific controls. The lower uptake of acetate by spores when partially purified exudates were added compared with the crude material (Fig. 2b) was mainly due to the methanol used to dissolve the partially purified root exudates. The treatment with methanol alone (Fig. 2b: methanol control; open squares) showed a lower uptake of acetate than the water control (Fig. 2b, open circles). By contrast, the uptake of glucose by germinating spores was stimulated by methanol (Fig. 2d). The presence of root exudates in the medium containing 10 μM glucose led to a threefold increase of uptake by the spores after 24 h, whereas the stimulatory effect on the uptake of acetate was much smaller (Fig. 2a,c). Part of the higher effect of root exudates on glucose uptake was caused by the lack of the prominent lag phase observed for the glucose uptake under control conditions (Fig. 2c). When root exudates were added to the medium, a significant uptake was detectable after 8 h, whereas under control conditions the first significant uptake was observed after 24 h.

In Fig. 3 the uptake, incorporation into biomass and respiration of both substrates are compared. Under control conditions (Ac CW) close to half of the acetate taken up by the spores was catabolized and released as CO_2. The amount of glucose (Glc CW) being used for respiration was lower because of the lower uptake by germinating spores. However, calculated as a fraction of the amount taken up, 46% of glucose also went into respiration. Both the uptake and the catabolic metabolism of substrates were significantly stimulated by both crude and partially purified root exudates. When root

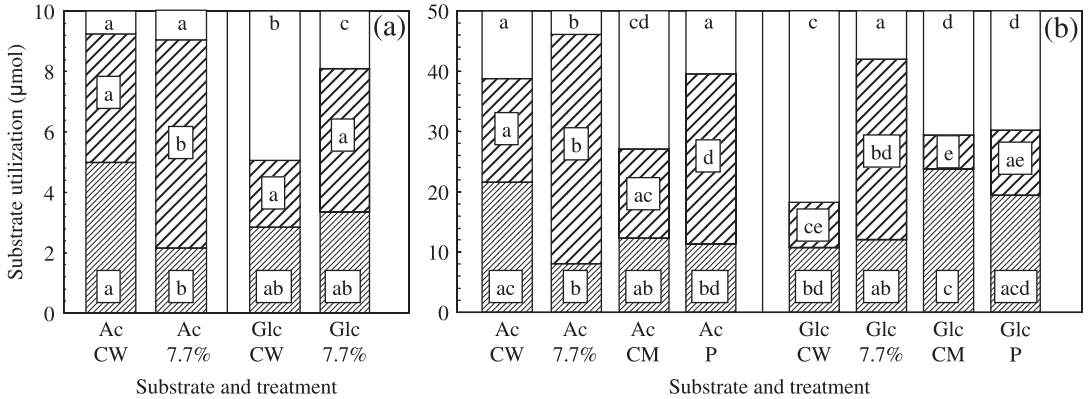

Fig. 3 Effects of root exudates on uptake, respiration and incorporation into biomass after supply of 10 μM (a) or 50 μM (b) acetate or glucose to germinating spores of *Glomus intraradices*. Residue in the medium (open bars, top), respiration (wide hatched bars, center) or biomass (narrow hatched bars, bottom). Means ($n = 8$) are shown. Different letters in the subdivisions of the bars (residue, respiration or biomass) indicate statistically significant differences between the treatments ($P \leq 0.05$). Ac, acetate; CM, control methanol; CW, control water; Glc, glucose; 7.77%, crude root exudates; P, partially purified root exudates.

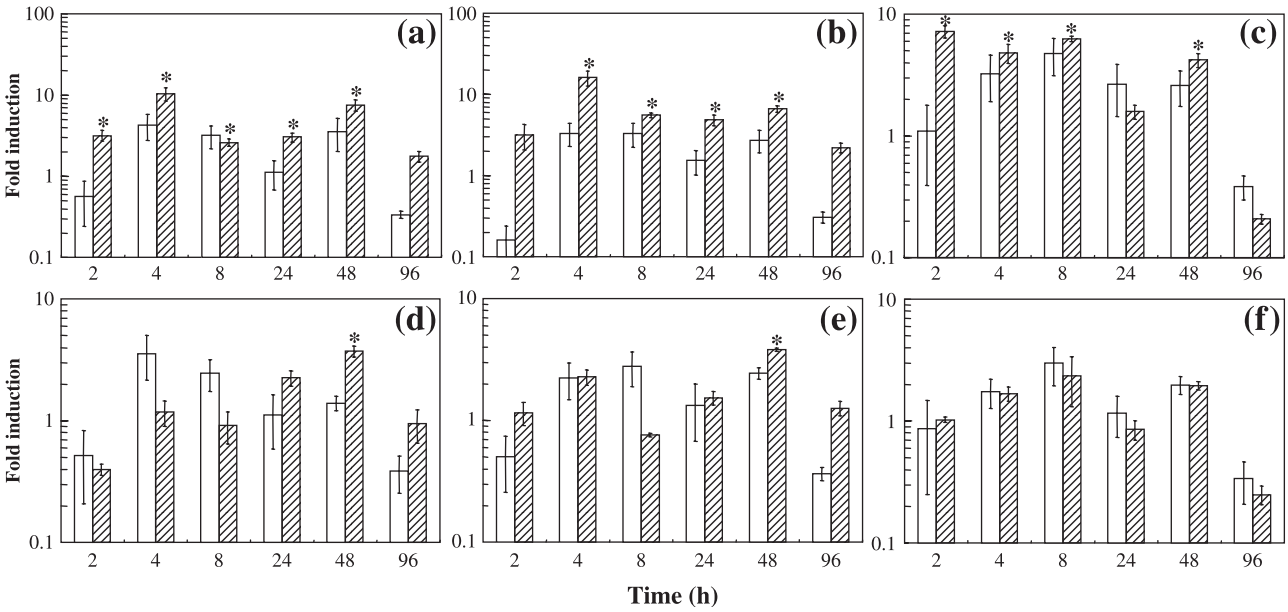

Fig. 4 Expression of putative genes involved in the C metabolism of *Glomus intraradices* after addition of crude (open bars) or partially purified root exudates (hatched bars) relative to the expression of SRP as reference gene. (a) Acyl-CoA dehydrogenase (ACD); (b) malate synthase (MS); (c) glutamine-fructose-6-P-aminotransferase (GFAT); (d) glycogen synthase (GLYS); (e) glucose-6-P-dehydrogenase (G6PDH); (f) trehalase (TE). Mean of three biological replicates ± SEM. The asterisks above the bars indicate a statistically significant stimulation of gene expression (all three biological replicates show a more than twofold increase in expression).

exudates were added to the medium containing 50 μM acetate, respiration was twice as high as in the respective controls (2.3-fold for crude, and 1.9-fold for partially purified root exudates) (Fig. 3b). The higher respiration when root exudates and acetate were added was not only because of a higher uptake but also a shift in metabolism towards catabolism at the expense of anabolism. When acetate was supplied, the incorporation into biomass was significantly reduced by the addition of root exudates (Fig. 3). By contrast, the increase in respiration of glucose by crude root exudates was mainly the result of higher glucose uptake, with little change in incorporation into biomass.

The conversion of carbon sources into the fungal storage sugar trehalose was not affected by root exudates. The ^{1}H-NMR spectra of extracts made after labeling germinating spores with [1-^{13}C]acetate or glucose for 6 d showed a threefold higher labeling in trehalose in the C_1 position when acetate was added (37.3 ± 2.2%, $n = 3 \pm$ SEM) compared with glucose addition (10.8 ± 3.0), but crude root exudates had no effect on the labeling (35.4 ± 1.4% or 10.3 ± 1.0%

after acetate or glucose addition, respectively). Partially purified exudates also did not increase the incorporation of labeled acetate into trehalose (22.3 ± 4.0% versus 22.7 ± 0.8% after incubation for 3 d with or without exudate addition). The labeling in fatty acids of storage lipids was low even after 14 d (0.65 ± 0.82%) and was also not affected by partially purified root exudates (1.21 ± 0.24%).

The effect of root exudates on fungal gene expression

Partially purified root exudates affected gene expression sufficiently to potentially alter the metabolism of germinating spores (Fig. 4). The expression of acyl-CoA dehydrogenase (ACD, Fig. 4a), malate synthase (MS, Fig. 4b), and glutamine-fructose-6-phosphate aminotransferase (GFAT, Fig. 4c) was increased by partially purified root exudates. The stimulation in gene expression was detectable within 2–4 h after adding exudates to the spores and remained substantially elevated up to 48 h and declined after 96 h. The transcript levels of glycogen synthase (GLYS, Fig. 4d), and glucose-6-P-dehydrogenase (G6PDH, Fig. 4e) were also slightly increased after 48 h, but not after 24 h or after 96 h so that the metabolic impact of this observation is unclear. By contrast, the expression of trehalase (TE, Fig. 4f) was not affected by root exudates. This result, together with the observation that root exudates did not stimulate the incorporation of labeled acetate into trehalose (see above) indicates that the trehalose carbon pool in germinating spores is not affected by root exudates. Crude root exudates, which had an effect on spore germination, hyphal branching and substrate uptake by germinating spores, did not significantly affect gene expression (Fig. 4).

The ICL protein levels during presymbiotic growth were slightly increased by crude root exudates (Fig. 5a,b) and this effect increased with the root exudate concentration in the medium (Fig. 5b). Partially purified root exudates also increased the ICL levels (Exu P) but the effect in this case did not differ significantly from the methanol control. An addition of 50 μM acetate to the medium had generally no effect on the ICL levels (Fig. 5b). A small increase of the ICL level was only found in the water control after the addition of acetate to the medium (Fig. 5b).

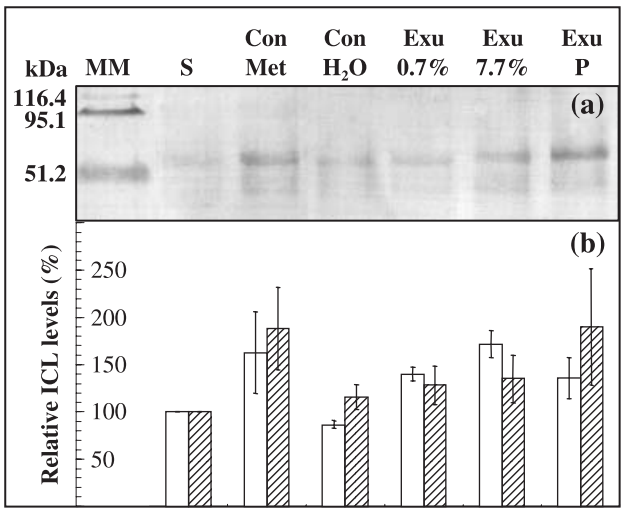

Fig. 5 Isocitrate lyase levels in germinating spores of *Glomus intraradices*. Western blot 24 h after addition of root exudates to spores in a medium containing 50 μM acetate (a) and quantification of the protein levels (b: open bars, control; hatched bars, medium with 50 μM acetate). Lanes from left to right: molecular marker (MM); S, spores at $t = 0$ (start of the experiment); control + methanol (Con Met); control (Con H_2O); Exu, crude root exudates at two concentrations (Exu 0.7% and Exu 7.7%); partially purified root exudates (ExuP).

Discussion

The root exudates were active and stimulated the presymbiotic growth of *G. intraradices*

The germination and branching assay confirmed that the crude root exudates that were used for the present studies on organic carbon uptake and metabolism were active and stimulated hyphal branching of *G. intraradices*. Many authors have reported increased hyphal growth as well as morphological changes such as the formation of highly branched structures as a response to root exudates (Bécard & Piché, 1989; Tarawaya *et al.*, 1996; Buée *et al.*, 2000; Nagahashi & Douds, 2000). The germination of the spores was also slightly accelerated by root exudates, but their presence in the medium was not required for germination. Despite their obligate biotrophy, AM fungal spores have been shown to be generally capable of spontaneous germination as long as certain physical and physiological conditions are fulfilled (Azcón & Ocampo, 1984; Bécard & Fortin, 1988; Logi *et al.*, 1998) and only slight effects of root exudates on spore germination have been described (Tarawaya *et al.*, 1996). When a host plant is absent, germ tube growth and development are limited and the fungal hyphae undergo a programmed growth arrest and resource reallocation, which maintains the viability and infection capability of the spores (Logi *et al.*, 1998).

The uptake of carbon sources by germinating spores is stimulated by root exudates

Germinating spores of *G. intraradices* are able to take up organic carbon compounds and an uptake of acetate could be observed within 2–4 h and was also maintained in the absence of host plant signals, when growth retardation occurred (Fig. 1a). This suggests that spores can remobilize their limited carbon reserves in the form of triacylglycerols (TAGs) or glycogen during germination (Pfeffer *et al.*, 1999), but can also maintain their metabolism with exogenous carbon resources, even when no host roots are present. The uptake and utilization of glucose and acetate by germinating spores

was also shown by Bago *et al.* (1999), but owing to the long labeling times (14 d) and high concentrations (4 mM acetate, 25 mM glucose) that were used in this study, no concentration and short-term effects could be identified. Acetate uptake was faster than the uptake of glucose and showed saturating kinetics with an apparent K_m (Fig. 1c) close to that of an acetate transporter that has been described in *Escherichia coli* and whose driving force is a transmembrane electrochemical potential (Gimenez *et al.*, 2003). However, the apparent K_m was determined over a period of days and therefore could also reflect the affinity of an internal metabolic process or processes.

Glucose uptake by germinating spores followed first-order kinetics (Fig. 1d), which could be interpreted as a passive uptake by diffusion across the membrane. However, the concentration range used in these experiments (0–50 μM) could have been too low to reach the saturation of a transporter. The K_m values of glucose transporters of other fungi are comparatively high. A monosaccharide transporter of the glomeromycotan fungus *Geosiphon pyriformis* has a K_m of *c.* 1.2 mM for glucose (Schüßler *et al.*, 2006). Transporters of *Saccharomyces cerevisiae* and the pathogenic fungus *Uromyces fabae* had K_m values of 360 μM or in the millimolar range, respectively (Özcan and Johnston, 1999; Voegele *et al.*, 2001). However, a hexose uptake transporter from the plant symbiotic ascomycete *Tuber borchii* showed after expression in *S. cerevisiae* a comparatively low K_m of 38 ± 10 μM for glucose (Polidori *et al.*, 2007). The prominent lag phase of 24 h found for the glucose uptake by germinating spores in these experiments and the lack of this lag phase when glucose is resupplied indicates the need for the induction of a transporter or of glucose-utilizing enzymes before uptake (Fig. 1b).

The assumption that acetate and glucose uptake by germinating spores may be coupled to a proton cotransport is consistent with the finding that the uptake of both was stimulated when root exudates were added to the spores (Fig. 2). Root exudates have been shown to stimulate the H^+ efflux and the H^+-ATPase activity of the fungal plasma membrane and the $^{32}P_i$ uptake by germinating spores of *Gigaspora margarita* (Lei *et al.*, 1991; Jolicoeur *et al.*, 1998; Ramos *et al.*, 2008). A hyperpolarization of the fungal plasma membrane as a response to root exudates was found almost immediately after the signal and the authors deduced that the first effects of interaction between plant and fungus occur via changes at the plasma membrane rather than via altered gene expression (Ayling *et al.*, 2000). The effects on uptake of exogenous carbon sources by germinating spores observed here were detected after short-term exposure to crude root exudates and these exudates had no effect on gene expression.

Crude root exudates in a low concentration had a higher effect on substrate uptake and catabolic activity than partially purified root exudates (Figs 2 and 3). This was at least partly due to the methanol used to dissolve the partially purified root exudates. This could result from an effect of methanol on uptake processes or on the activity of the root exudates. Casal *et al.* (1998) reported that the uptake of acetate by *S. cerevisiae* is reduced by ethanol and other alcohols and the authors suggested that alcohols interfere with the acetate carrier in the plasma membrane. It has also been shown that the activity of a strigolactone was drastically reduced by nucleophilic solvents such as methanol (Akiyama *et al.*, 2005). By contrast, partially purified root exudates dissolved in methanol have been shown to affect hyphal branching, mitochondrial activity and gene expression of germinating spores (Buée *et al.*, 2000; Nagahashi & Douds, 2000; Tamasloukht *et al.*, 2003; Lanfranco *et al.*, 2005; Besserer *et al.*, 2006). These considerations suggest that multiple plant compounds could be responsible for the regulation of presymbiotic metabolism and morphology in AM fungi.

Primary metabolic pathways in germinating spores are regulated by root exudates

Figure 6 summarizes the effect of root exudates on substrate uptake, metabolic processes and gene expression in germinating spores of *G. intraradices*. Partially purified root exudates stimulated a rapid increase in the expression of acyl-CoA dehydrogenase (ACD, Fig. 4a; process 1 in Fig. 6) which was sustained over 48 h. Triacylglycerol is the main storage form of carbon in spores and its breakdown by ß-oxidation fuels presymbiotic growth (Gaspar *et al.*, 1994; Bago *et al.*, 2002). Acetate uptake was also substantially stimulated by both crude and partially purified root exudates (Fig. 2a,b). Both processes, ß-oxidation and the conversion of acetate by acetyl-CoA synthetase (for review see Starai & Escalante-Semerena, 2004) provide acetyl-CoA as key substrate for the TCA cycle. This is also consistent with the stimulated catabolism of acetyl units to CO_2 observed here by both crude and partially purified root exudates via the TCA cycle (Figs 3 and 6). The respiration of acetate was not only stimulated by the higher uptake but also by a shift in metabolism towards catabolism at the expense of anabolism. The activation of the catabolic metabolism makes ATP available for active uptake processes (see above) and the biosynthesis can be sustained for hyphal growth required for root colonization. Partially purified root exudates and strigolactones have previously been shown to upregulate the expression of genes putatively involved in mitochondrial activity and the respiratory activity of germinating spores of different AM fungi (Tamasloukht *et al.*, 2003; Besserer *et al.*, 2006). By contrast, in the absence of a host plant, spores of AM fungi are able to germinate and it has been suggested that a low metabolic activity prevents the fungus from compromising its carbon and energy reserves entirely to maintain the viability and infection capacity (Bécard *et al.*, 2004). Spores of *Gigaspora gigantea* for example can germinate up to 10 times (Koske, 1981).

Lipid breakdown also supplies carbon skeletons for anabolic processes via the glyoxylate cycle and the activity of isocitrate lyase (ICL, process 2 in Fig. 6) and malate synthase (MS,

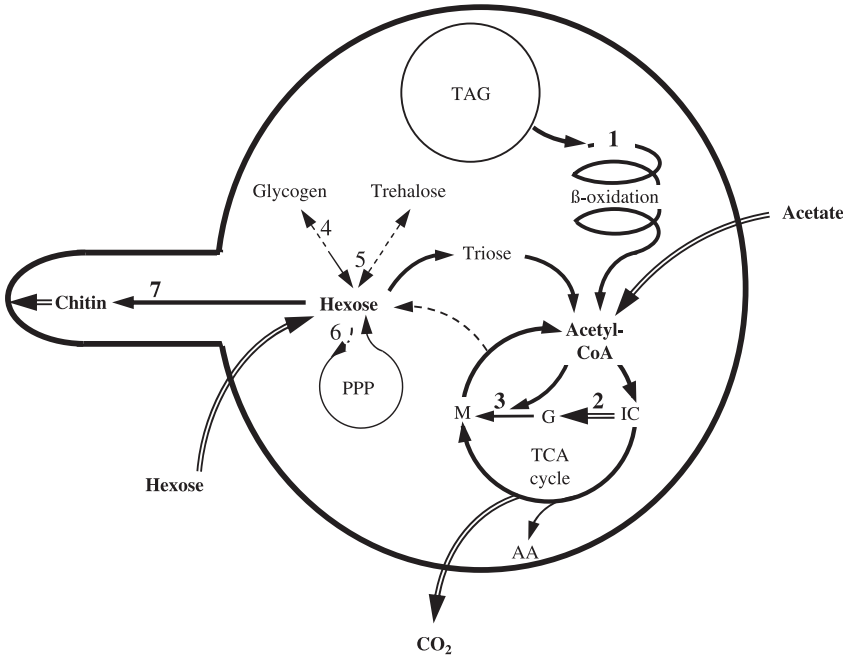

Fig. 6 Model of the pathways of carbon metabolism in germinating spores of *Glomus intraradices* and the effect of root exudates on these pathways. Processes stimulated by both crude and partially purified root exudates are indicated by double lines. Processes that are only stimulated by partially purified root exudates are shown in bold type. The following processes are shown: (1) breakdown of storage triacylglycerol (TAG) to acetyl-CoA by ß-oxidation (acyl-CoA dehydrogenase, Fig. 4a); glyoxylate cycle via (2) isocitrate lyase (Fig. 5) and (3) malate synthase (Fig. 4b); uptake of acetate and glucose (Fig. 2) respiration (Fig. 3); and (7) chitin biosynthesis (glutamine–fructose-6-P-transaminase, Fig. 4c). Processes not affected by root exudates: glycogen synthesis via (4) glycogen synthase (Fig. 4d); breakdown of trehalose via (5) trehalase (Fig. 4f); biosynthesis of trehalose (see text); pentose phosphate pathway (PPP) via (6) glucose-6-P-dehydrogenase (Fig. 4e).

process 3 in Fig. 6). Thus the stimulation of germ tube branching by root exudates is consistent with the higher transcript levels of MS (Fig. 4b, process 3 in Fig. 6) and glutamine-fructose-6-phosphate aminotransferase (GFAT, Fig. 4c, process 7 in Fig. 6) and the slightly increased protein levels of ICL (Fig. 5, process 2 in Fig. 6). However, despite the elevated expression of MS by partially purified root exudates, neither crude nor partially purified root exudates increased the incorporation of label from acetate into biomass and instead a lower labeling of the biomass was found (Fig. 3). This finding may result from dilution of label in the cytosolic acetyl-CoA pool owing to higher rates of lipid degradation (as indicated by the increased transcript levels of ACD; Fig. 4a, process 1 in Fig. 6) and/or to higher rates of utilization of acetate as a respiratory substrate (Fig. 3). The same dilution effect could also explain the lack of stimulation of incorporation of label from exogenously supplied glucose into biomass despite the increase in hyphal branching and increased GFAT transcript levels after addition of partially purified root exudates (Fig. 4c, process 7 in Fig. 6). However, the higher transcript levels of GFAT after root exudate addition were not sustained over the whole time course of the experiment (4 d) and returned to normal transcript levels after 48 h. This is consistent with the finding that the germination of spores was slightly accelerated and that only the hyphal branching but not the total fungal biomass is affected by root exudates.

Neither the labeling of trehalose after supplying ^{13}C-labeled glucose or acetate nor the expression of trehalase (TE, Fig. 4f, process 5 in Fig. 6) was stimulated by crude or partially purified root exudates. Trehalose is utilized during spore germination (Bécard *et al.*, 1991) but is also simultaneously synthesized when exogenous carbon sources are supplied (Shachar-Hill *et al.*, 1995; Bago *et al.*, 1999; Bago *et al.*, 2003). Higher metabolic flux rates from the hexose pool into the TCA cycle and into chitin biosynthesis could explain the finding that the labeling of trehalose was not increased by the addition of root exudates. The lack of higher transcript levels of TE by partially purified root exudates during spore germination is consistent with the finding that trehalose only represents a small part of the carbon storage in spores (between 0.06% and 1.6% of the spore dry weight; Bécard *et al.*, 1991).

An addition of partially purified root exudates did not lead to significant changes in the glycogen synthase transcript levels (GLYS, Fig. 4d, process 4 in Fig. 6). During spore germination and symbiosis, exogenously supplied carbon sources are converted to glycogen by AM fungi to buffer intracellular hexose levels and as a long-term storage in spores (Shachar-Hill *et al.*, 1995; Pfeffer *et al.*, 1999; Bago *et al.*, 2003). However, the lack of a significant flux from the hexose pool into glycogen via GLYS is also not surprising when host root signals are perceived by the spores and the utilization of internal carbon sources for catabolic processes is stimulated, especially under conditions where no exogenously supplied carbon sources were present. However, regulation of GLYS on a posttranslational rather than on a transcriptional level could also explain this effect. Bago *et al.* (2003) suggested that the GLYS activity is regulated post-translationally, based on the fact that various tissues in the symbiosis (ERM, IRM and spores) showed comparable transcript levels and that the amino acid sequence of GLYS contains putative phosphorylation and Glucose-6-phosphate allosteric regulatory sites (Pederson *et al.*, 2000).

The fungal hexose pool is also metabolized via the pentose phosphate pathway (PPP, Fig. 6) to produce pentose for the nucleic acid biosynthesis and/or to provide NADPH for

anabolic processes (Pfeffer *et al.*, 1999). When anabolic pathways in germinating spores are stimulated in response to root exudates, as indicated by the acceleration of germination and the increase of the GFAT transcript levels within the first 48 h of the time course (Fig. 4c, process 7 in Fig. 6), the higher demand for reducing equivalents could also drive the PPP in spores, but neither crude nor partially purified root exudates had a stimulatory effect on the gene expression of G6PDH at these early time-points (Fig. 4e, process 6 in Fig. 6). Only at 48 h, could a small increase in the G6PDH transcript levels be detected. The G6PDH transcript levels in the intraradical mycelium of *G. intraradices* have been shown to be regulated by the phosphorus availability for the host plant (Stewart *et al.*, 2006). The activity of G6PDH, however, is regulated mainly on a post-translational level in many other organisms (Hauschild & von Schaewens, 2003).

Conclusion

The results show that crude root exudates differ from partially purified root exudates in their effect on presymbiotic growth and metabolism of germinating spores. Crude root exudates that were supplied at comparatively low concentrations slightly accelerated spore germination and stimulated hyphal branching, substrate uptake and catabolic metabolism, but had no effect on gene expression. By contrast, partially purified root exudates led to higher transcript levels of ACD, MS and GFAT, but substrate uptake and respiration were not further affected by these concentrated root exudates compared with crude root exudates. The finding that changes in catabolic metabolism as a response to root exudates are not associated with significant changes in gene expression and vice versa could indicate that some of the processes are regulated at a post-translational rather than on a transcriptional level. The complex composition of root exudates, however, also suggests that multiple plant compounds could be responsible for the effects on presymbiotic growth, or that root exudates contain both stimulatory and inhibitory compounds. The observed differences in the effects of crude and partially purified root exudates could result from concentration-dependent effects, but also to changes in the composition after the purification process. Root exudates contain a variety of different compounds and whether a single compound or multiple plant signals trigger the responses of AM spores in the presymbiotic growth phase is still unknown, and needs to be further analysed.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 The effect of crude and partially purified root exudates on the expression of genes putatively involved in the C metabolism of germinating spores of *Glomus intraradices*

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