Supporting Online Material for

Reciprocal Rewards Stabilize Cooperation in the Mycorrhizal Symbiosis

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This PDF file includes:

Materials and Methods
SOM Text
Figs. S1 to S10
Tables S1 to S3
References
Supporting Online Material

Materials and Methods

Selection of fungal strains
We chose the three AM fungal species based on the following criteria: (1) all AM fungi belong to the same genus. By choosing closely related fungi, we were able to avoid problems associated with contrasting life history traits not necessarily associated with mutualistic benefit (27, 31, 32). (2) The fungi differentially affected growth of their host plant and this difference was evident within 10 weeks of growth (fig. S3). Although fungal benefits could potentially change (increase or decrease) over the host’s ontogeny (33-35), we were interested in documenting early-stage fungal and host allocation patterns, in which there were fewer constraints on fungal and plant growth. At this stage, clear allocation patterns are predicted because resources acquisition demand is at its highest (22). (3) The benefits conferred to hosts were consistent across different plant species (fig. S3). This allowed us to reject the hypothesis that the observed differences in mutualistic benefit were attributed to local co-evolutionary dynamics between host and fungal symbiont (36). (4) The selected AM fungi differed in growth benefit but were not ‘parasites’ (see 35, 37-39 for useful discussion). In our case the biomass of the plants inoculated with the less-cooperative AM fungal species was either equal, or greater than the growth of the non-mycorrhizal control plants (fig. S3). This allowed us to examine whether hosts could detect and respond to variation in fungal cooperation (40, 41), rather than testing for host response to a negative growth impact (e.g. a non-cooperative species (37)). (5) We utilized species with different structural patterns. At 10 weeks, G. custos allocated significantly more to spore production, and G. aggregatum allocated significantly more to vesicles compared to the other two AM fungal species (fig. S4). The use of these two less-cooperative species allowed us to test for host response when the choice was binary (G. intraradices versus G. aggregatum), and also test for host response in AM communities with three species, which included two less-cooperative species, G. aggregatum and G. custos differing in their carbon storage strategies. In these less-cooperative fungi, high spore and vesicle formation are potential indicators of fungal resource hoarding. Ratios of these fungal storage units to arbuscules (nutrient transfer structures) are often used as an estimate of symbiotic effectiveness (42-44). (6) Importantly, we do not categorize our less-cooperative species as unequivocal ‘cheats’ (38, 39, 45). AM fungi can confer diverse benefits to the host plant (protection against pathogens, drought, or heavy metal uptake) not measured here (46). It is well-known that biotic and abiotic changes can alter the relative benefits of AM fungi (47). No experimental design can explore all the diverse conditions under which the relationship with particular fungi is potentially beneficial (48). (7) To increase the ecological context of our experimental design, all fungal species were isolated from temperate ecosystems between 37-43° degrees, and from areas in which Medicago sp. hosts are found. While these species are globally cosmopolitan, it is well known that fungal isolates –within a species- can differ greatly in the benefits they confer to their hosts (49). While it would be interesting to conduct future experiments that utilize plant and fungal material collected from a single ecosystem, we note that there are difficulties in isolating fungal strains from one location that meet all our criteria for selection (see criteria 1-6 above).

Fungal cultures
For all experiments, we produced inoculum of Glomus intraradices (Schenck & Smith; isolate 09 collected from Southwest Spain by Mycovitro S.L. Biotecnologia ecológica, Granada, Spain), G. custos (Cano & Dalpé; isolate 010 collected from Southwest Spain by Mycovitro S.L.) and G. aggregatum (Schenck & Smith; isolate 0165 collected from the Long Term Mycorrhizal Research Site, University of Guelph, Canada) by growing the fungus in association with Ri T-DNA transformed carrot (Daucus carota clone DCI) roots in Petri dishes filled with mineral medium (50) and with sucrose as the only carbon source. We
cultured roots for approximately 8 weeks (until the plates were fully colonized) and fungal spores were isolated from the growth medium by solubilising the medium with 10 mM citrate buffer (pH 6.0).

**Design of species-specific quantitative real-time PCR (qPCR) markers, i.e. primers and hydrolysis probes**  
To quantify the abundance of each AM fungal species in the stable isotope probing (SIP) experiments, we designed markers targeting species-specific motifs in the mitochondrial large ribosomal subunit RNA genes of *G. intraradices*, *G. aggregatum* and *G. custos*.

**DNA preparation and amplification**  
We extracted fungal DNA from both spores and colonized roots produced monoxenically, as described below. We used DNeasy Plant Mini kit (Qiagen, Hombrechtikon, Switzerland) and followed the recommendations of the manufacturer with slight modifications. For spores only, the final volume of the DNA preparations was 20 µl (instead of recommended 100 µl) to maximize DNA concentration before PCR. DNA was subjected to PCR amplification of the mitochondrial large ribosomal subunit (mtLSU) RNA gene with following primer pair combinations, RNL11-RNL17, RNL1-RNL14, or RNL1-RNL15 (according to (51)). The PCR was carried out using Taq PCR Core kit with CoralLoad reaction buffer (Qiagen), using a 25 µl PCR reaction volume, 1 µM of each primer, and 38 cycles (denaturation at 95°C for 10 s, annealing at 50°C for 90 s and amplification at 72°C for 90 s). Amplified DNA fragments were cloned into a blue-script vector (pGEM-T Easy vector system; Promega, Dübendorf, Switzerland) and sequenced by Microsynth AG (Balgach, Switzerland). The sequences were individually edited and the clones re-sequenced if the quality of the reads proved to be insufficient. The identity of the sequences was revealed by BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to exclude potential contaminant sequences (e.g., bacteria, unspecific amplifications of other genome regions).

**Probe design**  
The sequences of our three AM fungal species were aligned with other available mtLSU sequences from e.g. *G. intraradices*, *G. proliferum* and *G. clarum* in order to construct our hydrolysis probes. For each fungal species at least two species-discriminating primers with associated hydrolysis probes were designed using the AlleleID software (version 6, Premier Biosoft International, Palo Alto, California, USA). Care was taken to target mtLSU regions coding for the ribosomal RNA to avoid putative introns described recently (52). We confirmed the specificity of the primers and fluorescent probes with a BLAST search and the oligonucleotides (primers and dually labeled hydrolysis probes, labeled with fluorescein at the 5’-end and BHQ-1 quencher at the 3’-end) were then synthesized by Microsynth AG (Balgach, Switzerland). Primers and probes were purified by preparative HPLC or preparative polyacrylamide gel electrophoresis, respectively, before lyophilization. Both primers and probes were diluted with PCR-grade water to achieve 25 µM concentrations, aliquoted (20 µl each) and frozen at -20°C.

**Primer selection, optimization of cycling conditions, cross-reactivity testing (DNA and cDNA)**  
To ensure species-level specificity, we performed several optimization steps. First, we tested the markers for specificity under low stringency cycling conditions (denaturation at 95°C for 10 s, annealing at 52°C for 30 s, and amplification at 72°C for 5 s). In this assay, we used DNA extracts from *M. truncatula* roots colonized by the different AM fungi (3 replicates for each species) as templates. From this initial test, primer pairs and probes showing greatest specificity towards their target species (either no cross-amplification with other species or the greatest difference in Cq value between target and non-target species) were selected for further optimization (see Table S1). Stringency of cycling conditions was then increased stepwise for each of the markers to avoid amplification of non-target samples (see Table S1 for details of the optimized cycling conditions and Table S2 for the results of the cross-
amplification assay). Finally, to confirm that the markers only amplified the target fungal species, and that they avoided plant genes and were suitable at the RNA level, we performed another cross-amplification assay using cDNA generated from RNA extracts of non-mycorrhizal or mycorrhizal roots of *M. truncatula* colonized by the different fungal species (Table S2). Again, all three markers were confirmed to be species-specific at both, DNA and RNA level.

**qPCR calibration and detection limits**

We generated plasmids carrying fragments of the mtLSU of the respective fungal species with 100% sequence match to the region amplified in order to: (1) to calibrate the qPCR detection cycle (Cq) with the gene copy concentrations and (2) to assess the detection limits of the qPCR markers. Cq is typically negatively and linearly correlated to the log-transformed template concentration (linear response region), until the detection limit of the assay is reached and the Cq becomes independent of the further dilution (background region) (Fig. S9), or there is no response at all. We used the linear response region of each calibration assay to derive equations that allowed the conversion of Cq values to mtLSU gene copies per unit volume of the template (Fig. S10). The detection limits were calculated from the background region of the qPCR response curve as follows:

\[
DL = AV_{Cq(back)} - 3 \times SD(AV_{Cq(back)})
\]

where DL represents the detection limit of the assay (Cq value), \(AV_{Cq(back)}\) the mean of the Cq values in the background region and \(SD(AV_{Cq(back)})\) the standard deviation of this mean. The detection limits of the three assays and the corresponding threshold concentrations of mtLSU are given below (Table S3). These assays were then used to determine the mtLSU gene copy concentration in DNA and cDNA samples, fractionated or not by ultracentrifugation, and taking into account any dilutions of the template during sample processing.

**Plasmid preparation**

Between two and four individual plasmid preparations per fungal species were used for the calibration of the qPCR markers. The plasmids were isolated from overnight cultures of transformed *E. coli* JM109 cells (Promega, Madison, WI, USA), grown on LB medium supplemented with 100 µg ml\(^{-1}\) Ampicillin, using the Miniprep procedure (33). The plasmids were linearised using the EcoRI digestion (Fermentas, Le Mont-sur-Lausanne, Switzerland) at 37°C for 2 h and then at 65°C for 20 min. The concentration of the DNA was then measured by the PicoGreen fluorescence assay (P7589, Invitrogen, San Diego, CA, USA), using Roche LightCycler 2.0 at 45°C and measuring the emission at 530 nm. The concentration of plasmid copies per unit of sample volume was calculated according to (54) under consideration of the DNA concentration in each sample, the length of the insert (176 bp for *G. intraradices*, 661 bp for *G. aggregatum*, and 438 bp for *G. custos*) and vector (3015 bp), and an estimated molecular weight per nucleotide double-stranded DNA of 660 Da. Plasmid preparations were serially diluted (5-fold and 10-fold) to achieve a range of plasmid concentrations from a few billions to (theoretically) less than 1 per microliter.

**Stable Isotope Probing**

**Plant culture**

Seeds of *Medicago truncatula* (variety Jemalong A17, courtesy of Bettina Hause, Leibniz Institute of Plant Biochemistry, Halle, Germany) were pre-treated with concentrated H\(_2\)SO\(_4\) and exposed to a cold treatment (4°C in the fridge) for 3 days. The seedlings were transferred to a sterilized peat-based growth medium for 5 days and then planted in 1 L pots filled with sterilized nutrient-poor dune sand with the following characteristics: pH 7.2; 0.2% organic matter; 0.3 mg kg\(^{-1}\) P(CaCl\(_2\)-extracted) and 190 mg kg\(^{-1}\) total N.
For the two-species experiment, the seedlings were inoculated at planting with 1500 spores per plant and 1.0 g of in vitro root material of either G. intraradices or G. aggregatum (singles) or both species together (mixed 50:50) with inoculum concentrations reduced by half. For the three-species experiment, G. custos was included in the mixed treatment and the inoculum concentrations of the three AM species were reduced to one-third each. We assumed that in this mixed treatment, the nutrient-acquiring strategies of our AM species were fixed, meaning that strategies did not undergo fundamental change (switch from less-cooperative to cooperative or vice versa) simply because other symbionts were present (55).

Non-mycorrhizal control plants were inoculated with autoclaved inoculum. Plants were grown in a greenhouse with a 13 h light cycle. When the outside daylight was below 120 J cm⁻² h⁻¹, supplemental lights of 15,000 lux, were turned on. The temperature was kept between 22 and 25°C. Soil humidity was maintained at 70% water holding capacity and nutrients (8 ml per pot of Hoagland solution (56) containing only 50% of original P concentration) were added every two weeks. The plants were grown for a total of 10 weeks before ¹³CO₂ labeling.

¹³CO₂ labeling and harvest
Plants were labeled with ¹³CO₂ at the Experimental Soil Plant Atmosphere System (ESPAS, Isolife, Netherlands) (57), with a day/night rhythm of 16/8 h and at 21 C and 17 C, respectively, an irradiation of 700 µmol m⁻² s⁻¹ at plant height, and 80% relative humidity. 

The plants were acclimated to the chamber for 48 h before labeling. The mean CO₂ level in the chamber was maintained at 401±19 µl l⁻¹ by injection of ¹²CO₂ from a pressurized cylinder. During the night period prior to labeling, ¹²CO₂ was removed by a CO₂-scrubber in accordance with the ¹²CO₂-respiration of M. truncatula. One hour before the start of the day period, ¹³CO₂ was injected from a pressurized cylinder (99 atom % ¹³C, 1 atom % ¹²C; Isotec, Inc. Miamisburg, OH, USA). For 6 h, a total CO₂-level (¹²CO₂ + ¹³CO₂) of 396±20 µl l⁻¹ ¹²CO₂ was maintained. The ¹³C-enrichment of the atmosphere was 92% at the start of the 6-h labeling period. This value gradually decreased due to the ¹²CO₂ respiration by the plant and resulted in a mean ¹³C-enrichment of 86.5±3.0 % over the time course of labeling.

In both the two-species and three-species experiment, the labeling chamber was opened and flushed with fresh air after 6 h to remove the labeled ¹³CO₂. After the flushing period, the labeling chamber was closed and the ¹²CO₂ level was maintained at 405±29 µl l⁻¹. To follow the incorporation of ¹³C label over time in the two-species experiment, replicate plants were harvested at the 6 h-flushing period and again 6 h later at the 12 h time point. In the three-species experiment an extra harvest time was added, so plants were harvested at 6 h, 12 h and 24 h. In both experiments, all replicates of the single inoculated control treatments were harvested at the 6 h time point. At each harvest, the aboveground plant parts were removed, oven dried at 70°C for 72 h, and weighed. The root systems were gently washed, weighed, homogenized and five root aliquots were placed in Eppendorf tubes and frozen with liquid N₂. A small subsample of roots was removed, processed in 10% KOH, and stained with trypan blue to quantify the mycorrhizal colonization and fungal structures in the root (58). Sand was collected and spores were counted using conventional decanting and wet sieving methods (59).

RNA extraction, ultracentrifugation, and cDNA synthesis by reverse transcription.
RNA was extracted from roots using the RNeasy Plant Mini Kit (Qiagen, Hombrechtikon, Switzerland), tested for quality and RNA concentration using a Nanodrop1000™ and stored at -80°C. For centrifugation, 500 ng of RNA was transferred in 2 ml ultracentrifuge tubes (Sysmex, Kobe, Japan) pre-filled with 1.99 ml of 1.8 g ml⁻¹ CsTFA solution. The samples were then placed into a Sorvall discovery m120 SE micro ultracentrifuge (Thermo Fisher Scientific, Waltham, MA, USA) with a S120VT fixed angle titanium vertical rotor for 48 h at 20°C at a speed of 64000 rpm, resulting in a gravity of 142,417 g at the maximum radius and
91,1128 g at the minimum radius. Between 17 to 20 fractions of 100 µl each were collected from every 2 ml vial. To remove these fractions, the tubes were punctured at the bottom and top using a needle. The upper needle was connected to a syringe pump (Harvard Apparatus, Kent, UK) that allowed a continuous flow rate (220 µL min⁻¹) of RNAse free water. This initiated a continuous flow of fractions from the lower needle. An extra vial was included in each ultracentrifugation batch for gravimetric estimation of density of each gradient fraction in each ultracentrifugation run (60). The RNA in each fraction was precipitated, dried and resuspended in 15 µl of ultrapure water. Five µl were then used for reverse transcription (RT), using a final volume of 220 µL min⁻¹ and the following reaction components: 5 µl 5xRT buffer, 1.5 µl of 10 mM dNTPs, 0.5 µl random hexamers, 1 µl of 200 u/µl, MMLV reverse transcriptase (Promega Corp., WI, USA) and 12 µl water.

Real time quantitative PCR (qPCR) analysis
All qPCR assays were carried out in 9 µl reactions, using the LightCycler 2.0 instrument, LightCycler TaqMan chemistry (LightCycler TaqMan Master) and 20 µl-Lightcycler glass capillaries. The final concentrations of the primers and the hydrolysis probe were 0.5 µM and 0.11 µM, respectively (for sequences see Table S1). Each reaction included 2.25 µl of the DNA template (i.e. sample).

Quantification of RNA abundance of the different fungal species
To quantify the enrichment of fungal RNA with host derived ¹³C in the different fractions, we used qPCR targeting species-specific sequence motifs in the mtLSU, as described above. All reactions were carried out separately, not multiplexed, under stringent cycling reaction (Table S1). Briefly, 2.25 µl of the RT reaction (see above) was used as a template for qPCR, and the total qPCR reaction volume was 9 µl. Gene copy concentrations were calculated per µl template using the quantification cycle (Cq) from each assay and the respective calibration curves (fig. S10). The results of mtLSU quantification of each AM fungal species in the different fractions were subjected to nonlinear regression, using the Gaussian, 3-parameter function option in SigmaPlot for Windows version 11.0. This function is described by the following formula:

\[ y = a \times e^{(-0.5 \times \left(\frac{x-x_0}{b}\right)^2)} \]

where a and b are constants, \(x_0\) is the x value of function peak, and e is the base of natural logarithm (approximately 2.718). Only the samples with \(R^2\) of all relevant regressions higher than 0.64 (i.e., \(R \geq 0.8\)) were used for subsequent statistical analyses. This data selection was necessary in order to exclude samples that suffered high RNA degradation during ultracentrifugation and subsequent steps, and/or poorly fractionated samples, where the gradients were obviously disturbed during fraction collection. This quality check resulted in the removal of 1 out of 12 samples in the two-species experiment, and 6 out of 17 samples in the experiment with three AM fungal species.

Analysis of peak front
Variation in host C allocation patterns were calculated based on differences in ‘peak front’ among AM species. Peak front is the position (i.e. density in mg ml⁻¹) of the heaviest RNA fraction of each of the AM fungal species. Each fungal species shows a unique peak front position that can be compared against the others. Peak front is defined mathematically as the foremost fraction of the Gaussian regression curves cutting through the detection limit of the qPCR assay. Peak fronts further to the left (see fig. S2 for example) mean higher ¹³C enrichment, indicative of preferential C allocation to that fungal species. To determine peak front differences among the AM fungal species within each individual plant sample, we first measured abundance of each AM fungal species (copies of mtLSU) in each RNA density fraction by using qPCR with species-specific markers (Table S1). Then, Gaussian regressions across the different fractions were constructed for each AM fungal species. Peak fronts for the different AM fungal species were compared only when meeting requirements listed above, thus removing technically imperfect samples from statistical comparison.
To determine if there were significant differences in $^{13}$C enrichment of our AM fungal species, we ran pair-wise comparisons of peak front position for all pairs of AM fungal species. We calculated differences in peak front positions based on a non-parametric sign test, using Statgraphics Plus software (version 3.1 for Windows). $P$-values (Fig. 1) refer to differences of the sample median from zero, with values above zero indicating preferential allocation to that particular fungal species.

To further confirm our preferential allocation findings, we ran additional analyses using a parametric generalized linear model (GLM) approach. For each replicate and each fungal species combination, differences in peak front positions between AM fungal species were calculated, as described above. A GLM was produced independently for both the two-species and three-species experiments to test the variables of differential $^{13}$C enrichment and harvest time. The Akaike criteria (AIC) was used to select the optimal GLM, which in our case was in the gamma family. A ‘saturated model’ reproduced the observed data. The relative importance of a given interaction term or a co-variable was estimated after removal of this term from the saturated model. Deviance analyses using Fisher tests were performed. Using this approach, we confirmed our finding that the RNA of the cooperative species ($G.$ intraradices) was significantly more enriched than that of the two less-cooperative species ($G.$ aggregatum and $G.$ custos). We found significantly higher $^{13}$C enrichment in both the two-species experiment ($G.$ intraradices vs. $G.$ aggregatum, $P = 0.019$) and in the three species experiment ($G.$ intraradices vs. $G.$ aggregatum, $P = 0.030$) and ($G.$ intraradices vs. $G.$ custos, $P = 0.016$). There was no significant difference in RNA enrichment of the two less-cooperative species ($G.$ aggregatum vs. $G.$ custos, $P > 0.05$). The GLM deviance analyses showed no significant effect of time on allocation patterns for both the two-species ($P = 0.4267$) and three-species ($P = 0.5571$) experiments. All GLM analyses were carried out using the program R (http://www.r-project.org/).

**Analyses of non-fractionated RNA samples**

The non-fractionated RNA samples were reverse transcribed and the cDNA was used as template for qPCR quantification of mtLSU copies as described above. The results were converted to mtLSU RNA copies per 500 ng RNA. These results were used to compare the abundance of the different fungal species in the roots after inoculation with single or mixed AM fungal species (fig. S5).

**Manipulation experiments with in-vitro root organ cultures**

For all resource manipulation studies, we used Ri-T-DNA-transformed carrot roots ($D.$ carota L., clone DC1), that were colonized with the cooperative AM fungus $G.$ intraradices or the less-cooperative AM fungus $G.$ aggregatum. These two fungal species were used for the resource manipulation experiments because they differed greatly in cost to benefit ratios for P to C exchange (~2.5 higher C costs in $G.$ aggregatum, Fig. 3A), and represented the maximum and minimum of the host benefit continuum ($6, 61, 62$).

While root organ cultures (ROCs) have been criticized for their artificial nature ($63$), it has been repeatedly demonstrated that ROCs possess similar nutrient and resource transfer and metabolic characteristics as whole plant systems ($64$). ROCs have been pivotal in producing a large body of literature that has shaped our understanding of nutrient transport and C exchange in the AM symbiosis ($65-73$). ROC model systems offer a number of important advantages for our study, including (1) the separation into fungal and root compartments (which prevented the diffusion and exchange of substrates between the compartments) and thereby precise control over quantities of resources supplied to fungus and host, (2) high visibility of the system, allowing us to select comparable plates for each experiment regarding e.g. the colonization of the fungal compartment, and (3) precision with which the ERM could be collected. In addition, ROCs provide the ideal model system for comparing particular traits (e.g. N or P transfer) across AM species, while standardizing for confounding environmental...
factors. This allowed us to compare baseline functioning and then manipulate resources to test for host and fungal responses to nutrient availability. Such small-scale manipulations are not yet possible in a soil based system. In the future, in-vitro whole-plant systems could be a useful test system for biological market experiments with AM fungi (74). However, the challenge of working with in-vitro whole plants is the loss of precision in controlling the carbohydrate allocation from the host to the fungus. Although manually shading plants can be utilized as a potential treatment to reduce host C, the effects are difficult to control and to quantify, and secondary effects of the reduced photosynthetic rate on plant physiology can not be excluded.

In vitro root organ cultures
We grew mycorrhizal systems in Petri dishes with two or three compartments (depending on the experimental design) at 25°C. The mycorrhizal roots were confined to one or two root compartments (50) filled with solidified mineral medium (75) containing 10 g l⁻¹ sucrose. AM fungi are obligate biotrophs that cannot use this C source directly but rely on carbon that is supplied by the host. After approximately eight weeks of growth, the colonized root compartments were transferred into new Petri dishes and the extraradical mycelium (ERM) of the fungus was allowed to cross over the divider into one or two fungal compartments (depending on the experimental design, see below). These fungal compartments were filled with solidified mineral medium without sucrose and phosphate addition (KH₂PO₄ was replaced with an equimolar concentration of KCl). After approximately 3 weeks, the fungal compartments were sufficiently colonized by ERM and the plates could be used for the experiments.

Experimental design of the ROC experiments
Preferential carbon transport from colonized roots to fungal ERM compartments differing in P supply.
Here, we asked the question: Will hosts transfer significantly more C to the fungal hyphae with access to more P (Fig. 2A,B)? We tested this question when hosts were colonized either by the cooperative species G. intraradices or the less-cooperative species, G. aggregatum. We used a three compartment Petri dish design with one mycorrhizal root compartment and two fungal compartments differing in P supply. Labeled sucrose (22.2 mM sucrose containing [U⁻¹⁴C]sucrose, 1:500000, v/v) with a specific activity of 498 mCi mM⁻¹ (Sigma-Aldrich, St. Louis, USA) was supplied to the root compartment. Simultaneously, water (0 µM P) was added to one fungal compartment and 35 µM P or 700 µM P (as KH₂PO₄) to the other fungal compartment. After 4 days, 6 replicates per treatment were harvested and processed for liquid scintillation counting (see below).

Preferential P transport from the ERM to root compartments differing in C supply.
Here, we asked the question: will significantly more P be transferred to the root compartment with access to more C (Fig. 2C,D)? Again, we tested this with the cooperative species G. intraradices and the less-cooperative species, G. aggregatum. We used a reciprocal design of the three compartment Petri dish system described above, now with two root compartments and one fungal compartment. This allowed us to track the transport of P from the fungal ERM to colonized roots that differed in their carbon supply. Fungal hyphae from both root compartments were allowed to cross over into one root-free compartment. When approximately the same number of hyphae had crossed over from each root compartment into the fungal compartment, 6.4 µCi [³²P]orthophosphate (Perkin Elmer, Waltham, USA) and 35 µM non-labelled KH₂PO₄ were added to the fungal compartment. The carbon supply in the root compartments was varied at the same time by adding 0.5 ml of water to one root compartment (0 mM control) and 0.5 ml of a sucrose solution to reach 5 mM or 25 mM in the other root compartment. After 4 days, 6 to 10 replicates per treatment were harvested and prepared for further analysis (see below).

Simultaneous measurements of symbiotic effectiveness and conditional response.
Here we asked two questions: (1) Does increasing host C supply lead to an increase in P transfer by both the cooperative and less-cooperative fungal symbionts (Fig. 3A) and (2) does the baseline cost to benefit ratios (here in terms of carbon costs for P supplied to the root) differ between the two fungal species (Fig. 3B)? To achieve both these aims, we used a two compartment Petri dish system with one root and one fungal compartment to which simultaneously $^{14}$C-sucrose and $^{32}$P-orthophosphate were added. Three weeks after the ERM started to cross over the divider, we added [U-$^{14}$C] sucrose with a specific activity of 498 mCi mM$^{-1}$ (Sigma-Aldrich, St. Louis, USA) to the root compartment and $[^{32}]$P orthophosphate (Sigma-Aldrich, St. Louis, USA) to the fungal compartment. To test for differences in P transport in response to increasing C supply and determine the cost to benefit ratio of each fungal species, one set of plates was only supplied with $^{14}$C labeled sucrose and $^{14}$C labeled sucrose diluted with non-labeled sucrose for a final sucrose concentration of 25 mM sucrose (0.448 μM as $^{14}$C labeled sucrose) was added to the other set. After 4 days, 8 replicates per treatment were harvested and prepared for further analysis (see below).

**Liquid scintillation counting**

For all experiments, we harvested the mycorrhizal roots and the fungal ERM after 4 days of labeling. The ERM was isolated from the medium in the fungal compartment after several wash and centrifugation steps in Na citrate buffer (10 mM, pH 6.0). An aliquot of the medium was taken to determine the radioisotope residues in the medium and to confirm that there were no cross-contaminations between compartments in the plates. The root and ERM samples were dried in an oven at 70°C, weighed and digested with a tissue solubilizer (TS-2, rpi corp., Mount Prospect, USA). The radioactivity was determined by liquid scintillation counting (Wallac, Perkin Elmer, Waltham, USA) using standard full channel programs in single isotope experiments or by channel settings that allowed the differentiation of $^{14}$C and $^{32}$P according to the emission energy in dual isotope experiments. The $^{14}$C measurements in the dual isotope experiments were additionally confirmed by measuring the samples for a second time 4 months later (i.e., after 8 half-lives of $^{32}$P passed), when $^{32}$P was sufficiently depleted. The accuracy of all measurements was corrected by use of an internal standard.

**Extraction of various phosphate pools and analysis of phosphate pool distribution**

To examine the phosphate pool distribution in mycorrhizal roots which were supplied with varying concentrations of sucrose (Fig. 2C,D, fig. S6, S7), we extracted phosphate pools according to the method described previously (76). The following phosphate pools were extracted and could be distinguished: (1) inorganic orthophosphate and acid soluble or short-chained polyphosphates (chain length of less than 20 P, residues) after extraction with 10 % TCA (w/v) at 4°C (two times); (2) phospholipids after extraction with first 100 % ethanol and then ethanol:ether (3:1, v/v), (3) acid insoluble or long-chained polyphosphates (chain length of more than 20 P, residues) after extraction with 1 M KOH at room temperature (two times), and (4) DNA-, RNA- and protein-phosphates (residue). Acid soluble polyP (short chain length) and acid insoluble polyP (long chain length) within the supernatants were precipitated two times by a saturated BaCl$_2$ solution over night at 4°C. We used polyP pools to measure P transport, because both polyphosphate pools are of fungal origin (plants are not able to produce polyP) and better represent P transport from the ERM to the IRM. The $^{32}$P content in all fractions was determined by liquid scintillation counting.

**Statistical Analysis**

Data from the ROC experiments were analyzed using Unistat Software, P-STAT Inc. (Hopewell, NJ, USA). For all experiments, the data were subjected to a variance analysis (ANOVA), with ‘resource-level’ as the treatment factor. Disintegrations per minute (dpm) values after scintillation counting were log transformed before the analysis. Following significant ANOVA, treatment means were compared using the Student-Newman-Keuls test ($P < 0.05$).
We conclude by raising three important points: (1) our work does not preclude the possibility that partners employ other mechanisms to control the growth/success of each other. Various mechanisms have been proposed to explain, for example, how mycorrhization may be mediated by host plants (4, 77-80). One possible mechanism is the digestion of fungal arbuscules by plant hosts (81). Although alternative explanations for premature arbuscular death cannot yet be ruled out (39), empirical work has demonstrated that the lifespan of an arbuscule may be related to its ability to deliver P (82) or to the P status of the host (83). Molecules such as lysophosphatidylcholine (LPC) have been suggested to be involved in P sensing and gene regulation in plants, potentially allowing hosts to evaluate the amount of P delivered via the mycorrhizal pathway (84). As more genome information becomes available, the molecular mechanisms governing the resource-sensing and control processes of both partners will be elucidated (85). (2) Here, we demonstrated the importance of P as a resource for determining trade dynamics (e.g. 86), but allocation based on other fungal commodities such as N, may likewise be important (87-89). Research is now needed to determine how resource stoichiometry (e.g. the relative availability of carbon, nitrogen and phosphorus) affects trade among partners. (3) Although our work demonstrated that trade is favored with partners offering the best rate of exchange, this finding does not imply equal control in the mutualism. It is well-known that at high P levels: (i) the mycorrhizal nutrient uptake pathway can be repressed (90), (ii) root exudate activity to stimulate presymbiotic growth of AM fungi is reduced (91), and (iii) the host may degrade the arbuscules of the fungus (81). In contrast, AM fungi are obligate biotrophs, meaning they will always rely on hosts for C. The implication is that, although fungi may choose to transfer P to the plant offering the highest C benefit, they will always need a host plant to complete their life cycle.
**Fig. S1:** Schematic drawing of the arbuscular mycorrhizal (AM) mutualism and resource exchange processes. (A) Land plants interact with diverse AM fungal communities (different species/strains represented by different colors) and AM fungi interact with multiple host plants. The mutualism is characterized by an exchange of mineral nutrients (e.g. N and P) from the fungus for C from the host plant. The transfer of nutrients occurs primarily across specialized structures called arbuscules (a). Fungal carbon is allocated to hyphae (h), vesicles (v) and/or spores (s). (B) Nutrient exchange between plant and fungal partner. Host C is transferred across the plant-fungal interface, taken up by the fungus and translocated to the extraradical mycelium (ERM). P is taken up from the soil as inorganic P (Pi) and converted into polyphosphates (PolyP). PolyP plays a key role in transferring nutrients to the intraradical mycelium. Nitrogen, as NH₄ and NO₃, is likewise absorbed from the soil by AM fungi, and assimilated mainly into arginine (Arg). PolyP are negatively charged polyanions that can also bind the basic amino acid Arg. In the intraradical mycelium, PolyPs are remobilized and release inorganic phosphate (Pi) and Arg. Arg is further broken down to inorganic N (specifically NH₄⁺), and then transferred across the plant-fungal interface.
Fig. S2: The detection of plant-derived C fluxes into microbial nucleic acids by stable isotope probing (SIP). (A) Plants were inoculated with three fungal species (red, blue, green). The plants were labeled with $^{13}$CO$_2$ that was then incorporated into the RNA of the AM fungal community. (B) After extraction, the fungal RNA was ultracentrifuged in a cesium trifluoroacetate gradient. (C) The ultracentrifugation fractionated the RNA in layers based on the relative amount of $^{13}$C-labeled carbohydrates incorporated by each fungal species. (D) Each centrifuge tube was punctured at the bottom and fractions (~18 per replicate) of 100 µL were taken using a long needle. The abundance of each AM fungal species was then quantified in every fraction using qPCR with species-specific markers targeting the mitochondrial large ribosomal subunit. (E) Results from the different fractions were then subjected to nonlinear regression analysis, and RNA buoyancy peaks for each fungal species within a replicate were plotted. Peak fronts, e.g. the position of the heaviest RNA fraction of each of the AM fungal species detectable by qPCR, were calculated. Peak fronts further to the left indicate a higher $^{13}$C enrichment in the fungal RNA (e.g. red peak front in the example shown). Peak front differences (delta values for RNA buoyancies in g ml$^{-1}$ of each pair of AM fungal species within each replicate) were determined and provided a paired species comparison of the C allocation patterns.
Fig. S3: Growth benefits conferred by the three AM fungal species and non-mycorrhizal (NM) controls. There was a significant effect of inoculation treatment in both the dicot and monocot plant species, (A) Medicago truncatula (F$_{3,65}$ = 52.808, $P < 0.001$) and (B) Allium porrum (F$_{3,58}$ = 4.494, $P = 0.007$). In M. truncatula, inoculation with the cooperative species (G. intraradices) led to a significant growth benefit compared to both less-cooperative species (G. aggregatum and G. custos) (Tukey’s honestly significant difference (HSD), $P \leq 0.05$). These results were confirmed with the monocot A. porrum. G. intraradices again led to significantly higher growth than G. aggregatum or G. custos (Tukey’s HSD, $P \leq 0.05$). In both plant species, the less-cooperative strains were not ‘parasites’, meaning colonization by these fungal species lead to either greater (M. truncatula) or equal (A. porrum) growth compared to the NM-controls. This allowed us to examine whether hosts could detect and respond to variation in fungal cooperation, rather than testing for host response to a negative growth impact. Letters indicate significant differences between treatments means according to Tukey’s HSD test ($P \leq 0.05$). Bars represent the means of 15 replicates ± 1 standard error.
**Fig. S4**: Mycorrhizal growth characteristics of the three AM fungal species. All three species colonized more than 80% of the host root length of *M. truncatula* when grown alone, however structural patterns differed significantly among species. (A) The less-cooperative species *G. aggregatum* formed significantly less arbuscules per root length than the other two species ($F_{2,44} = 6.917, P = 0.003$). (B) *G. aggregatum* formed significantly more vesicles per root length than the other two species ($F_{2,44} = 110.599, P < 0.001$). (B) The less-cooperative species *G. custos* invested significantly more in spores compared to the other two fungal species ($F_{2,26} = 18.747, P < 0.001$). Data were log transformed before analysis to meet assumptions for variance homogeneity. Different letters indicate significant differences between treatments means according to Tukey’s HSD test ($P \leq 0.05$). Figures (A) and (B) show the means of 15 replicates $\pm 1$ standard error. Figure (C) shows the mean of 9 replicates $\pm 1$ standard error.
**Fig. S5:** Changes in the abundance of different AM fungal species in association with *M. truncatula*, when alone or in mixtures (e.g. equal proportions of all three species). Abundance of AM species was assessed by species-specific qPCR on cDNA prepared from non-fractionated RNA samples. There was no significant difference in the abundance of *G. intraradices* when the plant was inoculated with *G. intraradices* alone or in mixture ($F_{1,8} = 0.05, P = 0.84$). In contrast, there was a significant decrease in the abundance of *G. aggregatum* ($F_{1,8} = 6.39, P = 0.035$), and *G. custos* ($F_{1,8} = 63.6, P <0.001$), when compared to their singly inoculated controls. Cochran’s C Test and Bartlett’s test indicated no major deviation from the null hypothesis of equal variance between treatments. Bars represent the means of n=3-7 ± 1 standard error. Asterisks indicate significant differences between treatment means.
**Fig. S6:** Long-chain PolyP pools of cooperative and less-cooperative AM fungi in a one-fungal, two-root compartment experiment. The less-cooperative *G. aggregatum* transferred more P to the root system that was better supplied with C, but retained the P in the form of long-chained polyphosphates (PolyP) (92), a form unavailable for the host (93). This could represent a potential hoarding strategy (see also fig. S7, S8). In contrast, the cooperative fungus *G. intraradices* converted a larger proportion of its long-chained PolyP to short-chained PolyP. Short-chained PolyP are continuously broken down in the intraradical mycelium to orthophosphate, which is transferred across the mycorrhizal interface to the host plant, and represent the PolyP pool that is correlated to host plant benefit (93,94). Long-chained PolyP concentrations were higher in roots that were colonized with the less-cooperative AM fungus *G. aggregatum* compared to roots colonized with *G. intraradices*, both in (A) dpm mg⁻¹ root dry weight (5 mM F₁,₁₃ = 4.42; *P* = 0.055 and 25 mM F₁,₁₃ = 6.10; *P* = 0.026) and (B) in % of total PolyP (5 mM F₁,₁₄ = 10.051; *P* = 0.0068 and 25 mM F₁,₁₃ = 5.404; *P* = 0.0369). The bars represent the mean of n= 6 to 9 replicates ± 1 standard error. Asterisks indicate significant differences between species within each sucrose treatment.
**Fig. S7:** The less-cooperative *G. aggregatum* retained significantly more P in form of long-chained polyphosphates (PolyP), than the cooperative AM fungus *G. intraradices*. As in the triple-plate experiment (fig. S6), *G. aggregatum* retained the P in the form of long-chained PolyP. The differences were not significant when no sucrose was added to the root system (0 mM; $F_{1,13} = 0.907, P = 0.341$), but significant when 25 mM sucrose was added to the root system and more carbon became available for the fungus ($F_{1,8} = 12.682; P = 0.0074$). The bars represent the mean of $n=5$ or 8 replicates ± 1 standard error. Asterisks indicate significant differences between species within each sucrose treatment.
Fig. S8: Model showing carbon and phosphate exchange in roots colonized with a cooperative (left) or less-cooperative AM fungus (right). The host root allocates carbon preferentially to the cooperative AM fungus (Fig. 1), which invests C resources into structures for increasing nutrient uptake and exchange, such as chitin for the extension of the hyphae (e.g. extraradical mycelium, ERM) in the soil. This allows the cooperative AM fungus to absorb more inorganic orthophosphate ($P_i$) from the soil and to transfer more P to the host (69, 95). The phosphate is transferred in the form of long-chained polyphosphates (PPP$_i$, dark grey) to the intraradical mycelium (IRM) (96). Here, the cooperative fungus breaks down long-chained polyP into short-chained polyP ($PP_i$, light grey) (figs. S6, S7) and then to inorganic orthophosphate ($P_i$). Short-chained polyP represents a relatively mobile polyP pool (97), while long-chained polyP represents a long-term storage pool of phosphate (93, 94). This remobilization to short-chained polyP is likely facilitated by higher C conditions in the IRM (69). The increase in the P$_i$ pool in the IRM facilitates the efflux into the interfacial apoplast and the uptake by the plant from the apoplast via mycorrhiza-specific P transporters (82, 98). In contrast, the less-cooperative AM fungus invests more carbon resources, such as triacylglycerides (TAG) (99) into the development of spores and vesicles (fig. S4), and less into the development of nutrient absorbing ERM. Phosphate that is transferred to the IRM of the less-cooperative fungus is stored mainly in the form of long-chained polyP, and conversion to short-chained polyP is low (figs. S6B, S7). This reduces the inorganic phosphate pool in the fungal cytoplasm and reduces the efflux of P through the fungal plasma membrane into the mycorrhizal interface that is driven by the concentration gradient between the fungus and the host (69, 100-102). Storage of P in a long-chained form can be advantageous because it allows the fungus to better control the transfer of P across its plasma membrane by reducing P efflux. Hoarding of P resources also potentially reduces P availability for competing fungi and any P that is directly available for host uptake, making the host plant more dependent on the mycorrhizal pathway for its nutrients (35, 39). However, fungal P hoarding also results in higher carbon costs for P for the host when the plant is P deficient, and has no choice in fungal partners (Fig. 3). The different strength of the arrows indicates higher or preferential fluxes (bold) and lower or reduced fluxes (thin).

Abbreviations: ERM - extraradical mycelium, IRM - intraradical mycelium, P$_i$ - inorganic phosphate, PP$_i$ - short-chained polyphosphates, PPP$_i$ - long-chained polyphosphates, TAG - triacylglycerides.
Fig. S9. Response of the qPCR signal (quantification cycle, Cq) to DNA template dilutions. Here, the intra mt5 marker for the DNA preparation of *G. intraradices* is shown. For the calibration of the qPCR assay only values of the linear response region were used. The background region was used to determine the detection limit of the qPCR assay.
Fig. S10: Calibration curves for the qPCR assays. Curves were designed to assess abundance of AM fungal species with markers targeting species-specific sequence motifs of the mitochondrial large ribosomal subunit (mtLSU) of (A) *G. intraradices*, (B) *G. aggregatum* and (C) *G. custos*. The calibration was carried out with serially diluted plasmid preparations carrying the respective DNA fragments. Equations for the conversion of the qPCR signal (i.e., quantification cycle, Cq) to the gene copy concentrations in the template are given for each assay. CP represents the number of target gene copies per µl template.
Supplementary Tables S1-S3

Table S1: qPCR markers for specific quantification of development of *Glomus intraradices*, *G. aggregatum*, and *G. custos* by measuring gene copies of the mitochondrial large ribosomal subunit of the respective AM fungal species. *FAM* – fluorescein, *BHQ1* – fluorescence quencher.

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<th>Annealing (°C / s)</th>
<th>Amplification (°C / s)</th>
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<td>60 / 10</td>
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<td>95 / 10</td>
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Table S2: Results of cross-specificity assay under optimized (stringent) cycling conditions for each AM species-specific qPCR marker. For templates, we used DNA extracts from spores and roots, as well as cDNA preparations from root RNA extracts. Sample provenance gives the information where the sample was produced, not where the nucleic acids were extracted and/or processed. All the qPCR analyses were carried out in Eschikon, Switzerland, using the same Roche LightCycler 2.0 instrument and Roche TaqMan chemistry. ROC – root organ culture, nd – no signal detected, n.a. – not applicable, BLD – below detection limit of the particular marker system.

<table>
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Table S3: Detection limits and minimal detectable target gene concentrations of the three qPCR assays.

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References

26. Materials and methods are available as supporting material on Science Online.