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Reciprocal Rewards Stabilize Cooperation in the Mycorrhizal Symbiosis

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Plants and their arbuscular mycorrhizal fungal symbionts interact in complex underground networks involving multiple partners. This increases the potential for exploitation and defection by individuals, raising the question of how partners maintain a fair, two-way transfer of resources. We manipulated cooperation in plants and fungal partners to show that plants can detect, discriminate, and reward the best fungal partners with more carbohydrates. In turn, their fungal partners enforce cooperation by increasing nutrient transfer only to those roots providing more carbohydrates. On the basis of these observations we conclude that, unlike many other mutualisms, the symbiont cannot be "enslaved." Rather, the mutualism is evolutionarily stable because control is bidirectional, and partners offering the best rate of exchange are rewarded.

The symbiosis between plants and arbuscular mycorrhizal (AM) fungi is arguably the world's most prevalent mutualism. The vast majority of land plants form AM interactions, in which plants supply associated AM fungi with carbohydrates, essential for fungal survival and growth (*1*). In exchange, AM fungi provide their host plants with mineral nutrients [e.g., phosphorus (P)] and other benefits such as protection against biotic (pathogens and herbivores) and abiotic (e.g., drought) stresses (*2*). This partnership, which evolved long before mutualisms among insects or vertebrates (*3*), is credited with driving the colonization of land by plants, enabling massive global nutrient transfer and critical carbon sequestration (*2*, *4*).

The selective forces maintaining cooperation between plants and AM fungi are unknown (*3*–*7*). Providing nutritional benefits can be metabolically costly, leading to the expectation that partners may defect from mutualistic duties (*6*, *8*). If individual host plant and fungal symbiont interests are tightly aligned (*9*), fungal symbionts will increase their own fitness by helping plants grow (*10*), and vice versa. However, plants are typically colonized by multiple fungal species (*11*), and fungal "individuals" can simultaneously interact with multiple host plants (*12*) or species (fig. S1) (*13*). This can select for "cheaters" that exploit the benefits provided by others while avoiding the costs of supplying resources (*3*, *8*). It is possible that plants have evolved mechanisms to enforce cooperation by fungi, analogous to the sanctions against uncooperative partners demonstrated in diverse mutualisms (*14*–*17*). However, sanction mechanisms in other systems appear to rely on a single host interacting with, and controlling the fate of, multiple partners. In contrast, the AM symbiosis involves a complex series of many-to-many interactions with multiple fungal strains (*11*) and multiple hosts (*13*), and it is not clear whether sanctions could operate in the same way.

An alternative explanation for the stability of the plant-mycorrhizal mutualism is that both plants and fungi are able to detect variation in the resources supplied by their partners, allowing them to adjust their own resource allocation accordingly. Such exchange of resources, in economic terms, represents a "biological market," in which

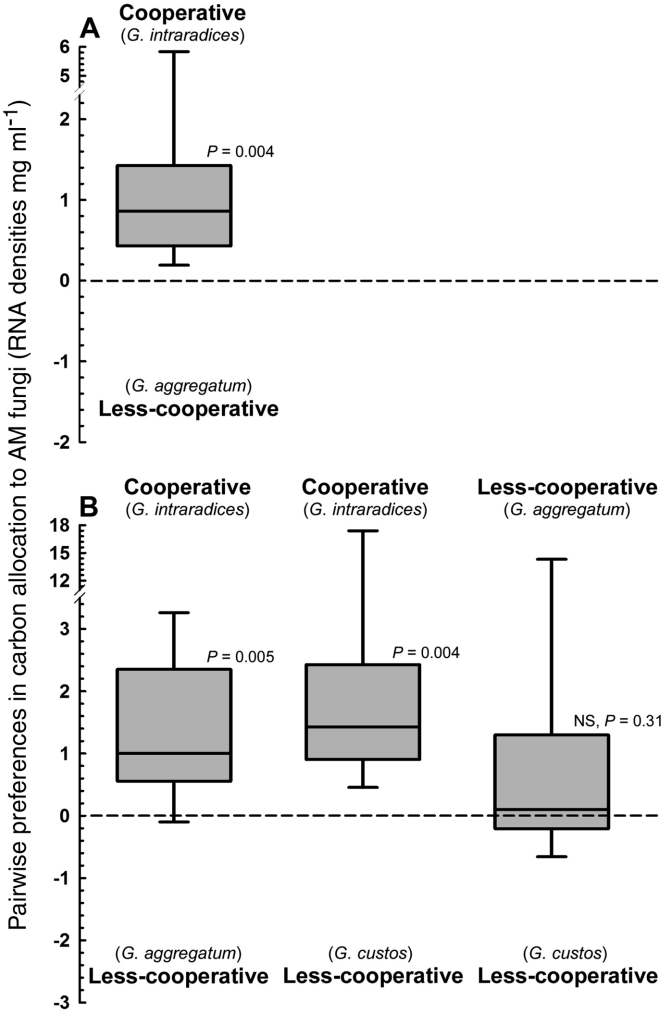

Fig. 1. Pair-wise comparisons of carbon allocation patterns to coexisting AM fungal species based on ^{13}C enrichment. Values above the zero line indicate preferential allocation to species above the line. (**A**) More carbon was allocated to the cooperative species (*G. intraradices*) compared with the less-cooperative species (*G. aggregatum*) in a two-species experiment. (**B**) When host plants were colonized with three AM fungal species, the RNA of the cooperative species (*G. intraradices*) was again significantly more enriched than that of the two less-cooperative species (*G. aggregatum* and *G. custos*). There was no significant difference in RNA enrichment between the two less-cooperative species. Data from all harvest times were pooled because there was no significant effect of time on RNA enrichment (Kruskal-Wallis, $P > 0.05$ for all three fungal species). Middle lines of box plots represent median values ($n = 11$), with bars showing value ranges (minimum to maximum). *P* values refer to nonparametric sign tests for differences of sample median from zero.

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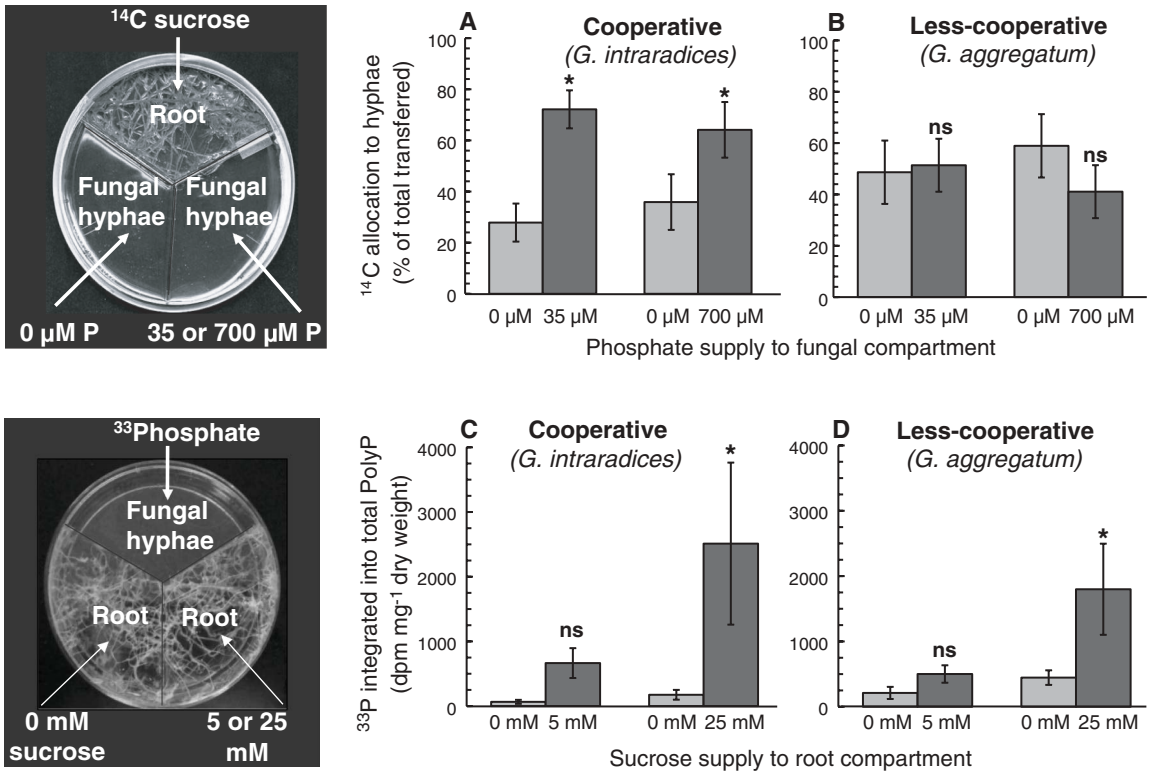

Fig. 2. Triple-plate experiments to mimic partner cooperation or defection. We found a significant effect of P availability on C allocation patterns ($F_{3,20} = 5.29$, $P = 0.0075$), with preferential allocation of C to the fungal compartments with access to more P in (**A**) *G. intraradices* but not in (**B**) *G. aggregatum*. In the reciprocal experiment, we found a significant effect of the C availability on P allocation patterns ($F_{7,58} = 7.298$, $P < 0.0001$), with a higher allocation of fungal P [measured as polyphosphate (PolyP)] to root compartments with higher C in both (**C**) *G. intraradices* and (**D**) *G. aggregatum*. However, the less-cooperative species *G. aggregatum*, remobilized a smaller percentage of its long-chained PolyP into short-chained PolyP, indicative of a hoarding strategy (figs. S6 and S8). Asterisks indicate significant differences between treatment means (Student-Newmans-Keuls test, $P \leq 0.05$). Error bars represent the means of 8 to 10 replicates ± 1 SEM.

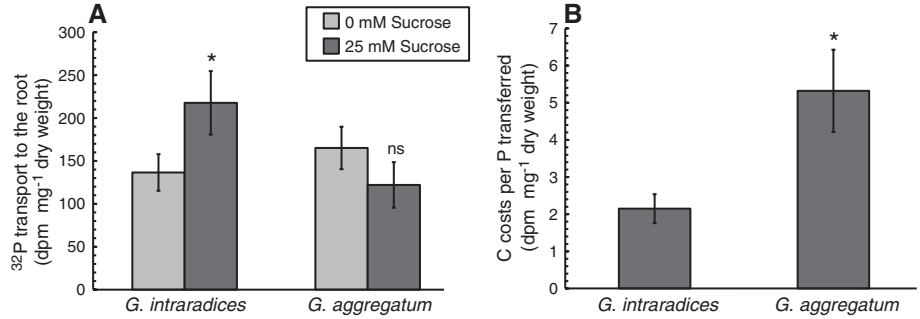

Fig. 3. Simultaneous measurement of P and C exchange. (**A**) Higher C availability stimulated increased P transfer by the cooperative species, *G. intraradices* ($F_{3,22} = 3.07$, $P = 0.0489$) but not by the less-cooperative species, *G. aggregatum*. (**B**) When supplied with 25 mM sucrose, the carbon costs per root P of *G. aggregatum* were more than twice as high as with *G. intraradices* ($F_{1,11} = 8.27$, $P = 0.0151$). Dpm, disintegrations per minute. Asterisks indicate significant differences between treatment means (Student-Newman-Keuls test, $P \leq 0.05$). Error bars represent means of 6 to 8 replicates ± 1 SEM.

partners exchange commodities to their mutual benefit (*18*–*21*). However, while mutualism market analogies have a strong theoretical basis (*19*, *22*, *23*), plants may be unable to discriminate among intermingled fungal species on a fine enough scale to reward individual fungi (*24*). Empirical tests have previously been constrained by our inability to track host resources into diverse AM assemblages and by difficulties in manipulating the cooperative behavior of both fungal and plant partners.

We resolved these constraints by allowing fungal genotypes that differ in their cooperative behavior to compete directly on a single root system. We used stable isotope probing (SIP) to track and quantify plant resource allocation to individual fungal species (fig. S2) (*11*) and hence test for host discrimination against less-cooperative partners. We also employed in vitro root organ culture approaches (*25*) to manipulate cooperative behavior of both plant and fungal mutualists to examine patterns of reciprocal rewards in response to variable levels of cooperation (*26*).

We used the model plant *Medicago truncatula* and three arbuscular mycorrhizal fungal species within the cosmopolitan subgenus Glomus Ab (*Glomus intraradices*, *G. custos*, and *G. aggregatum*). These AM fungi exhibited either high or low levels of cooperation (symbiont quality), based on plant growth responses, costs of carbon per unit P transferred, and resource hoarding strategies, with the two less-cooperative species directing more carbon resources either into storage vesicles (*G. aggregatum*) or spores (*G. custos*) compared with the cooperative species (figs. S3 and S4). We used closely related species to avoid potential confounding factors attributed to differences in life history traits not linked to nutrient exchange (*27*). We do not categorize our less-cooperative species as unequivocal "cheats," noting that they may confer other benefits not measured here (*26*).

We grew *Medicago* hosts with one, two (*G. intraradices* versus *G. aggregatum*), or all three AM fungal species. We followed the C flux from the plant to the fungal partners by tracking plant-assimilated C after 6 hours in a $^{13}CO_2$ atmosphere (*11*). We harvested the roots after 6, 12, and 24 hours to follow the incorporation of host carbon into the RNA of the AM fungal assemblage. We focused on RNA because it better reflects immediate C allocation patterns relative to DNA (*28*). Total RNA extractions were then subjected to ultracentrifugation to separate fractions based on the level of ^{13}C incorporation. By quantifying mitochondrial ribosomal RNA transcripts via specifically designed primers and quantitative polymerase chain reactions (qPCRs), we were able to track the real-time relative C allocation to each of the AM fungal species (figs. S2, S9, and S10).

We found that more carbon was supplied to the more-cooperative fungal species. In both the two-species and three-species experiments, the RNA of the cooperative fungus, *G. intraradices*, was significantly more enriched with host ^{13}C than the RNA of both less-cooperative species of the same genus (Fig. 1). We reject the hypothesis that the less-cooperative species were simply incompatible partners because colonization in all single-species controls were above 80% (fig. S4). Moreover, we found a significant effect of host preference on fungal abundance. *G. aggregatum* decreased by 36.7% ($F_{1,8} = 6.39$, $P = 0.035$) and *G. custos* by 85% ($F_{1,8} = 63.6$, $P < 0.001$) in communities where a high-quality partner was available (fig. S5), suggesting either a shift in resource supply by the host to the more-cooperative species or changes in competitive dynamics among the fungi (*26*).

The extent to which cooperation can be effectively enforced depends on the scale at which hosts discriminate against less-cooperative fungal symbionts. For plant hosts, this detection would have to occur at a very fine spatial scale (e.g., ~1 cm or smaller), because genetically distinct fungi can form closely intermingled networks within host root systems (*1*). However, it has been argued that plants cannot discriminate among mixed fungi once colonization has been established (*24*). Discrimination based on fungal signaling before colonization is unlikely because there is no reason that fungi would have to signal honestly (*3*).

To resolve this potential paradox, we investigated whether fine-scale host discrimination occurs between fungal hyphae colonizing the same host root. We used an in vitro triple split-plate system, with one mycorrhizal root compartment and two fungal compartments composed of the same fungal species but varying in P supply. This allowed us to mimic cooperation or defection by fungal partners connected to the same host root and to track how this influences C allocation back to the fungus (Fig. 2, A and B). If hosts rely on nutrient transfer as a tool to discriminate between partners on the same root (*6*, *7*), we would predict higher C allocation to the hyphae with access to higher P resources.

We found that hosts rewarded fungal hyphae that were supplied with greater P resources. As predicted, 4 days after the addition of ^{14}C-labeled sucrose to the root compartment, we found that significantly more C was transferred to the fungal hyphae with access to more P (Fig. 2A). In the cooperative species, *G. intraradices*, even small quantities of available P (e.g., 35 μM) resulted in a 10-fold increase in C allocation to the hyphae, relative to the hyphae with no access to P. We found no C allocation differences when hosts were colonized by the less-cooperative species, *G. aggregatum* (Fig. 2B).

Like their plant hosts, AM fungi interact with multiple partners in nature (*13*). Consequently, fungi may also enforce cooperation by rewarding increased C supply with greater P transfer. Therefore, we used a reciprocal triple split-plate experimental design, with one fungal and two root compartments, to determine whether the fungal partner would preferentially allocate P to the host providing more carbohydrates (Fig. 2, C and D). We found that the cooperative species transferred more P to roots with greater access to C resources (Fig. 2C), confirming that fungi can discriminate among hosts differing in C supply. In contrast, the less-cooperative species, *G. aggregatum*, responded differently. Like the cooperative species, it transferred more P to the root compartment with access to more C, showing that it was able to assess and respond to the rate of C supply (Fig. 2D). However, this species predominantly stored the P resources in long-chained polyphosphates, a host-inaccessible form (fig. S6) (*29*). This type of resource hoarding potentially reduces P availability for competing fungi and P directly available for host uptake (fig. S8) and illustrates key differences in fungal strategies, with *G. intraradices* being a "reciprocator" and *G. aggregatum* a less-cooperative "hoarder."

To track simultaneous resource exchange between partners, and hence determine whether AM fungi are stimulated to provide more P in direct response to a greater host C supply, we used a two-compartment Petri plate design. Host roots were exposed to labeled U-^{14}C sucrose in either high or low concentrations, and labeled ^{32}P was added to the fungal compartment. We found that increasing C supply stimulated P transfer by the cooperative fungal species *G. intraradices* but not the less-cooperative species *G. aggregatum* (Fig. 3A). As above, the cooperative species responded to C rewards with a reciprocal P increase, whereas the less-cooperative species stored P in the host-inaccessible form of long-chained polyphosphates (fig. S7). Finally, we compared the ratio of C costs to P transferred in both species (Fig. 3B), confirming that colonization by the less-cooperative species resulted in significantly higher host costs. These results support our whole plant SIP experiments (Fig. 1) and explain why the plant host consistently allocated more C to the cooperative species when given a choice.

Overall, our results suggest that stability of the AM mutualism arises in a different way compared with other mutualisms. A general feature of many mutualisms is that one partner appears to be "in control" (*30*) and has either domesticated the other partner (*9*) or enforces cooperation through punishment or sanction mechanisms (*3*). In these cases, the potential for enforcement has only been found in one direction, with the controlling partner housing the other partner in compartments, which can be preferentially rewarded or punished, such as in legume root nodules (*16*), fig fruits (*17*), and the flowers of yucca (*15*) and *Glochidion* plants (*14*). In contrast, in the mycorrhizal mutualism, both sides interact with multiple partners, so that neither partner can be "enslaved." Cooperation is only stable because both partners are able to preferentially reward the other. This provides a clear, nonhuman example of how cooperation can be stabilized in a form analogous to a market economy, where there are competitive partners on both sides of the interaction and higher quality services are remunerated in both directions (*18*–*20*).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/333/6044/880/DC1
Materials and Methods
SOM Text
Figs. S1 to S10
Tables S1 to S3
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