Assigned Reading:

Glycan Foraging in Vivo by an Intestine-Adapted Bacterial Symbiont

Coevolution of yeast mannan digestion: Convergence of the civilized human diet, distal gut microbiome, and host immunity

Rhizobial and Mycorrhizal Symbioses in *Lotus japonicas* Require Lectin Nucleotide Phosphohydrolase, Which Acts Upstream of Calcium Signaling
These soft tissues are preserved as carbon tissues (7) stained (3) and are similar in morphology to fixed ostrich osteocytes, both unstained (Fig. 4D) and stained (3) for better visualization (Fig. 4D, inset). SEM verifies the presence of the features seen in transmitted light microscopy, and again, projections extending from the surface of the microstructures are clearly visible (Fig. 4E and F).

The fossil record is capable of exceptional preservation, including feathers (4–6), hair (7), color or color patterns (7, 8), embryonic soft tissues (9), muscle tissue and/or internal organs (10–13), and cellular structure (7, 14–16). These soft tissues are preserved as carbon films (4, 5, 10) or as permineralized three-dimensional replicas (9, 11, 13), but in none of these cases are they described as still-soft, pliable tissues.

Mesozoic fossils, particularly dinosaur fossils, are known to be extremely well preserved histologically and occasionally retain molecular information (6, 17, 18), the presence of which is closely linked to morphological preservation (19). Vascular microstructures that may be derived from original blood materials of Cretaceous organisms have also been reported (14–16).

Pawlicki was able to demonstrate osteocytes and vessels obtained from dinosaur bone using an etching and replication technique (14, 15). However, we demonstrate the retention of pliable soft-tissue blood vessels with contents that are capable of being liberated from the bone matrix, while still retaining their flexibility, resilience, original hollow nature, and three-dimensionality. Additionally, we can isolate three-dimensional osteocytes with internal cellular contents and intact, supply filopodia that float freely in solution. This T. rex also contains flexible and fibrillar bone matrices that retain elasticity. The unusual preservation of the originally organic matrix may be due in part to the dense mineralization of dinosaur bone, because a certain portion of the organic matrix within extant bone is intracrystalline and therefore extremely resistant to degradation (20, 21). These factors, combined with as yet undetermined geochemical and environmental factors, presumably also contribute to the preservation of soft-tissue vessels. Because they have not been embedded or subjected to other chemical treatments, the cells and vessels are capable of being analyzed further for the persistence of molecular or other chemical information (3).

Using the methodologies described here, we isolated translucent vessels from two other exceptionally well-preserved tyrannosaurs (figs. S1 and S2) (3), and we isolated microstructures consistent with osteocytes in at least three other dinosaurs: two tyrannosaurs and one hadrosaur (fig. S3). Vessels in these specimens exhibit highly variable preservation, from crystalline morphs to transparent and pliable soft tissues.

The elucidation and modeling of processes resulting in soft-tissue preservation may form the basis for an avenue of research into the recovery and characterization of similar structures in other specimens, paving the way for micro- and macro-taphonomic investigations. Whether preservation is strictly morphological and the result of some kind of unknown geochemical replacement process or whether it extends to the subcellular and molecular levels is uncertain. However, we have identified protein fragments in extracted bone samples, some of which retain slight antigenicity (3). These data indicate that exceptional morphological preservation in some dinosaurian specimens may extend to the cellular level or beyond. If so, in addition to providing independent means of testing phylogenetic hypotheses about dinosaurs, applying molecular and analytical methods to well-preserved dinosaur specimens has important implications for elucidating preservational microenvironments and will contribute to our understanding of biogeochemical interactions at the microscopic and molecular levels that lead to fossilization.

Glycan Foraging in Vivo by an Intestine-Adapted Bacterial Symbiont

Justin L. Sonnenburg,1,2 Jian Xu,1,2 Douglas D. Leip,1,2 Chien-Huan Chen,1,2 Benjamin P. Westover,1,3 Jeremy Weatherford,1,3 Jeremy D. Buhrer,1,3 Jeffrey I. Gordon1,2*

Germ-free mice were maintained on polysaccharide-rich or simple-sugar diets and colonized for 10 days with an organism also found in human guts, Bacteroides thetaiotaomicron, followed by whole-genome transcriptional profiling of bacteria and mass spectrometry of cecal glycans. We found that these bacteria assembled on food particles and mucus, selectively induced outer-membrane polysaccharide-binding proteins and glycoside hydrolases, prioritized the consumption of liberated hexose sugars, and revealed a capacity to turn to host mucus glycans when polysaccharides were absent from the diet. This flexible foraging behavior should contribute to ecosystem stability and functional diversity.

The adult human body is a composite of many species. Each of us harbors ~10 times as many microbial cells as human cells (1). Our resident microbial communities provide us with a variety of metabolic capabilities not encoded in our genome, including the ability to harvest otherwise inaccessible nutrients from our diet (2). The intestine contains an estimated 10 trillion to 100 trillion microorganisms that are largely members of Bacteria but include representatives from...
Archaea and Eukarya (1, 3). Changes in diet appear to produce only modest effects on the species composition of the adult human colonic microbiota, although its metabolic activity may change considerably (4). The genomic foundations of these metabolic adjustments have yet to be defined in vivo.

Fermentable carbohydrates are the principal energy source for human colonic anaerobes (4). Recent studies of Escherichia coli, a facultative anaerobe with relatively minor representation in the microbiota, revealed that genetic restriction of its ability to metabolize various monosaccharides differentially compromised its ability to colonize the intestines of mice that had undergone antibiotic “knockdown” of their microbiota (5). The stability of an ecosystem apparently correlates with the ability of its members to mount diverse responses to environmental fluctuations; a corollary is that adaptive foraging behavior stabilizes ecosystems (6). Here, we use a simplified gnotobiotic mouse model of the human intestinal ecosystem to show that Bacteroides thetaiotaomicron, a highly abundant obligate anaerobe found in the colonic microbiota of most normal adult humans (7, 8), redirects its carbohydrate-harvesting activities from dietary to host polysaccharides according to nutrient availability.

*B. thetaiotaomicron* is a glycolophile that can break down a broad array of dietary polysaccharides in vitro [reviewed in (3)]. This capacity is reflected in its genome (9), which has the largest repertoire of genes involved in acquisition and metabolism of polysaccharides among sequenced microbes, including 163 paralogs of two outer-membrane proteins that are site of great microbial density and diversity in conventionally raised mice (12). Nutrient use by *B. thetaiotaomicron* in the cecum was defined initially by whole-genome transcriptional profiling. Cecal contents, including the mucus layer, were removed immediately after killing of nonfasted mice (n = 6), and RNA was extracted (11). The *B. thetaiotaomicron* transcriptome was characterized with the use of custom GeneChips containing probe pairs derived from 4719 of the organism’s 4779 predicted genes (table S1) (11). The results were compared to transcriptional profiles obtained from *B. thetaiotaomicron* grown from early log phase to stationary phase in a chemostat containing minimal medium plus glucose (MM-G) as the sole fermentable carbohydrate source (fig. S1).

Unsupervised hierarchical clustering of the GeneChip data sets disclosed remarkable uniformity in the in vivo transcriptional profiles of *B. thetaiotaomicron* harvested from individual gnotobiotic mice (fig. S2A). A total of 1237 genes were defined as significantly up-regulated (11) in vivo relative to their expression in MM-G. The functions of these up-regulated genes were classified by clusters of orthologous groups (COG) analysis. The largest up-regulated group belonged to the “carbohydrate transport and metabolism” COG, whereas the largest group of genes down-regulated in vivo belonged to the “amino acid transport and metabolism” COG (fig. S3A).

The starch utilization system (Sus) proteins SusC and SusD are components of a *B. thetaiotaomicron* outer-membrane complex involved in binding of starch and malto-oligosaccharides for subsequent digestion by outer-membrane and periplasmic glycoside hydrolases (13). Thirty-seven SusC and 16 SusD paralogs were up-regulated in vivo by a factor of ≥10 relative to bacteria growing in MM-G (fig. S4A).

The indigestibility of xylan-, pectin-, and arabinose-containing polysaccharides in dietary fiber reflects the paucity of host enzymes required for their degradation. The human genome contains only one putative glycoside hydrolase represented in the nine families of enzymes known in nature with xylanase, arabinosidase, pectinase, or pectate lyase activities, whereas the mouse genome has none (10). In contrast, *B. thetaiotaomicron* has 64 such enzymes (table S2) (10), many of which were selectively up-regulated in vivo by factors of 10 to 823. These included five secreted xylanases, five secreted arabinosidases, and a secreted pectate lyase (Fig. 2, A to C) (fig. S4B).

GC-MS analysis of total cecal contents harvested from fed germ-free mice (11) revealed that xylene, galactose, arabinose, and glucose were the most abundant monosaccharide components (Fig. 2D). After 10 days of colonization by *B. thetaiotaomicron*, significant reductions in cecal concentrations of three prominent hexoses (gluco-
cose, galactose, and mannose) were observed. There were no significant decreases in pentoses or amino-sugars (Fig. 2D). The selective depletion of hexoses likely reflects the combined effects of microbial and host utilization. *B. thetaiotaomicron* colonization increased expression of the principal sodium/glucose transporter Sglt1 in the intestinal epithelium, reflecting an enhancement of host utilization of liberated monosaccharides (14). Moreover, of the 1237 bacterial genes up-regulated in vivo, 310 were assign able (11) to Enzyme Commission (EC) numbers in metabolic maps in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (15). The results of this metabolic reconstruction were consistent with active delivery of mannose, galactose, and glucose to the glycolytic pathway, and arabinose and xylose to the pentose phosphate pathway [fig. S5; see (16) for maps of all *B. thetaiotaomicron* genes with EC assignments that exhibited changed expression in vivo versus MM-G].

Host mucus provides a “consistent” endogenous source of glycans in the cecal habitat that could offer alternative nutrients to the microbiota.
during periods of change in the host’s diet. *B. thetaiotaomicron* embeds itself in this mucus layer (Fig. 1D). GeneChip analysis provided evidence that the bacterium harvests glycans from mucus. For example, in vivo, *B. thetaiotaomicron* exhibited significant up-regulation (by factors of 2 to 10; *P* < 0.05) of (i) an operon (BT0455-BT0461) that encodes a sialidase, sialic acid–specific 9-O-acetylesterase, mannosidase, and three β-hexosaminidases (Fig. 2A); (ii) a mucin-desulfating sulfatase (BT3051); and (iii) a chondroitin lyase (BT3350). Fucose in host glycans is an attractive source of food: It typically occupies a terminal α-linked position and is constitutively produced in the cecal mucosa (17).

We found that two secreted α-fucosidases (BT1842, BT3665) and a five-component fucose utilization operon (BT1272-BT1277) were induced (Fig. 2A). Operon induction, which occurs through the interaction of 1-fucose with a repressor encoded by its first open reading frame (ORF) (18), is indicative of bacterial import and utilization of this hexose.

To determine whether the absence of fermentable polysaccharides in the diet increases foraging on mucus glycans, we compared *B. thetaiotaomicron* gene expression in the ceca of two groups of age- and gender-matched adult gnotobiotic mice. One group received the standard polysaccharide-rich chow diet from weaning to killing. The other group was switched to a diet devoid of fermentable polysaccharides but rich in simple sugars (35% glucose, 35% sucrose) 14 days before colonization. All mice were colonized with *B. thetaiotaomicron* for 10 days, and bacterial gene expression was defined in each of their ceca at the time of killing.

The presence or absence of polysaccharides in the diet did not produce a significant effect on the density of cecal colonization (19). Using the transcriptional profiles of 98 *B. thetaiotaomicron* genes from the “replication, recombination, and repair” COG as biomarkers, we found that the cecal bacterial populations clustered most closely to cells undergoing logarithmic growth in vitro, irrespective of the diet (fig. S2B and table S3). The simple-sugar diet evoked a *B. thetaiotaomicron* transcriptional response predominated by genes in the “carbohydrate transport and metabolism” COG (fig. S3B). Glycoside hydrolase and polysaccharide lyase genes that were up-regulated in vivo by a factor of ≥2.5 relative to MM-G cultures segregated into distinct groups after unsupervised hierarchical clustering (Fig. 3) (fig. S6). The group of 24 genes most highly expressed on the simple-sugar diet encoded enzymes required for degradation of host glycans (e.g., eight hexosaminidases, two α-fucosidases, a sialidase) and did not include any plant polysaccharide-directed arabinosidases or pectin lyases. In addition, all components of the fucose utilization operon (BT1272-BT1277) were expressed at higher levels in mice fed the simple-sugar diet (average induction greater than MM-G by a factor of 12) than in mice fed the polysaccharide-rich diet (average induction greater than MM-G by a factor of 6). The sialylated glycan degradation operon (BT0455-BT0461) exhibited a comparable augmentation of expression on the simple-sugar diet.

A similar cluster analysis revealed two distinct groups of genes encoding carbohydrate binding/importing SusC/SusD paralogs: a group of 61 expressed at highest levels in *B. thetaiotaomicron* from the ceca of mice fed a polysaccharide-rich diet, and a group of 21 expressed at highest levels with a simple-sugar diet (fig. S7). Thirteen of the SusC/SusD paralogs expressed at highest levels on a polysaccharide-rich diet are components of predicted operons that also contain ORFs specifying glycoside hydrolases and polysaccharide lyases. Five pairs of the SusC/SusD paralogs expressed at highest levels on a simple-sugar diet are part of predicted operons. No SusC/SusD paralogs from one diet group are found in operons containing up-regulated glycoside hydrolase genes from the other diet group (fig. S8). Together, the data indicate that subsets of *B. thetaiotaomicron’s* genome are dedicated to retrieving either host or dietary polysaccharides (depending on their availability), although it appears that when both sources are available, harvesting energy from the diet is preferred.

Diet-associated changes in glycan-foraging behavior were accompanied by changes in the expression of *B. thetaiotaomicron’s* capsular polysaccharide synthesis (CPS) loci (fig. S9). Relative to growth in MM-G, CPS3 was down-regulated in vivo irrespective of host diet, CPS4 was up-regulated in the ceca of mice fed a polysaccharide-rich diet, and CPS5 was up-regulated with a high-sugar diet (fig. S9). The

**Fig. 3.** Diet-associated changes in the in vivo expression of *B. thetaiotaomicron* glycosome hydrolases and polysaccharide lyases. Unsupervised hierarchical clustering yields the following groups of genes up-regulated in vivo by a factor of ≥2.5 on average, relative to their average level of expression at all growth phases in MM-G: group 1, highest expression on a simple-sugar diet, includes activities required for degradation of host glycans; group 2, equivalent expression on both diets; group 3, highest on a polysaccharide-rich standard chow diet; includes enzymes that degrade plant glycans. Average relative differences in expression in vivo versus in vitro (MM-G) are shown in fig. S6. Predicted enzyme substrate specificities unique to the group are listed at the right.
Introduction

Predators Transform Subarctic Islands from Grassland to Tundra

D. A. Croll,1,* J. L. Maron,2 J. A. Estes,1,3 E. M. Danner,1 G. V. Byrd4

Top predators often have powerful direct effects on prey populations, but whether these direct effects propagate to the base of terrestrial food webs is debated. There are few examples of trophic cascades strong enough to alter the abundance and composition of entire plant communities. We show that the introduction of arctic foxes (Alopex lagopus) to the Aleutian archipelago induced strong shifts in plant productivity and community structure via a previously unknown pathway. By preying on seabirds, foxes reduced nutrient transport from ocean to land, affecting soil fertility and transforming grasslands to dwarf shrub forb-dominated ecosystems.

Nearly half a century ago, Hairston et al. (1) proposed that plant productivity and composition were influenced by apex predators through cascading trophic interactions. According to their “Green World” view, the direct effects of predators on herbivore populations transcend multiple trophic levels indirectly to enhance plant community productivity and biomass. Despite great progress in food web ecology, the indirect effects of top predators on vegetation dynamics in terrestrial systems remain unresolved and actively debated (2–6). Compelling demonstrations of multitrophic predator impacts on entire plant communities are scarce, in part because the spatial and temporal scales necessary to perform the appropriate community-wide experiments are daunting.

The introduction of predators to islands provides an opportunity to explore the indirect effects of predators on vegetation. Introduced predators commonly have devastating direct effects on their prey (7). The histories of these introductions are often well known, and the relative simplicity and isolation of insular systems facilitate the study of whole-community responses. Here we investigate how the introduction of arctic foxes (Alopex lagopus) to the Aleutian archipelago affected terrestrial ecosystems across this 1900-km island chain.

The Aleutian archipelago is a remote series of physically similar volcanic islands extending westward from the Alaska Peninsula (Fig. 1). The archipelago currently supports 29 species of breeding seabirds, together numbering >10 million individuals (8). Seabirds deliver nutrient-rich guano from productive ocean waters (9) to the nutrient-limited plant communities (10, 11). Historically, seabirds inhabited most islands along the Aleutian chain. Following the collapse of the maritime fur trade in the late 19th and early 20th centuries, foxes were introduced to >400 Alaskan islands as an additional fur source (12). The introduced foxes severely reduced local avian faunas, especially seabirds (13). However, several islands remained fox free, either because introductions failed or were not undertaken (12–14). Hence, a large-scale natural experiment to evaluate the effects of exotic predators on insular ecosystems was unwittingly initiated more than a century ago. We use this experiment to show how differing seabird densities on islands with and without foxes affect soil and plant nutrients; plant abundance, composition, and productivity; and nutrient flow to higher trophic levels. These determinations
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Coevolution of yeast mannan digestion: Convergence of the civilized human diet, distal gut microbiome, and host immunity

D Wade Abbott 1,*, Eric C Martens 2,*, Harry J Gilbert 3,*, Fiona Cuskin 3, and Elisabeth C Lowe 3

1Lethbridge Research Center; Agriculture and Agri-Food Canada; Lethbridge, Alberta, Canada; 2Department of Microbiology and Immunology; University of Michigan Medical School; Ann Arbor, MI USA; 3Institute for Cell and Molecular Biosciences; The Medical School; Newcastle University; Newcastle upon Tyne, UK

The complex carbohydrates accessible to the distal gut microbiota (DGM) are key drivers in determining the structure of this ecosystem. Typically, plant cell wall polysaccharides and recalcitrant starch (i.e. dietary fiber), in addition to host glycans are considered the primary nutrients for the DGM; however, we recently demonstrated that α-mannans, highly branched polysaccharides that decorate the surface of yeast, are also nutrients for several members of Bacteroides spp. This relationship suggests that the advent of yeast in contemporary food technologies and the colonization of the intestine by endogenous fungi have roles in microbiome structure and function. Here we discuss the process of yeast mannan metabolism, and the intersection between various sources of intestinal fungi and their roles in recognition by the host innate immune system.

Keywords: catabolism, carbohydrate active enzyme, distal gut microbiota, evolution, fungal cell wall, mannooligosaccharide, polysaccharide, symbiosis, yeast mannan

*Correspondence to: D Wade Abbott; Email: wade.abbott@agr.gc.ca; Eric C Martens; Email: emartens@umich.edu; Harry J Gilbert; Email: harry.gilbert@ncl.ac.uk

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The ‘sugar-coated’ surface of yeast

Yeast mannan is synthesized onto a GlcNAc2-Man8–9 acceptor, a highly conserved core oligosaccharide in eukaryotic glycoproteins, in the Golgi apparatus by a series of specific glycosyl transferases. The first steps involve the M-Pol I complex, which contains 2 family 62 glycosyl transferases Mnn9p and Van1p. Mnn9p catalyzes the first reaction and primes Man8GlcNAc2 with a single α-1,6 decoration, which is then extended into a highly conserved backbone, comprising ~200 Man units, by Van1p. This backbone is decorated by species-specific sidechains. For example, S. cerevisiae sidechains contain Manα-1,3→Manα-1,2→Manα-1,2→Man with occasional phosphomannoligosaccharide branches; whereas the invasive pathogen Candida albicans displays Manβ-1,2 oligosaccharides of varying length that cap the α-linked Man side chains (Fig. 1). These terminal structures represent 2 of the growing list of signature carbohydrate structures that are being linked to fungal perception and host immunity.
Orchestrated metabolism of yeast mannann by the DGM

The human genome contains only a handful of CAZymes known to be active on dietary carbohydrates, and the majority of those are involved in the digestion of α-glucans (e.g., isomaltooligosaccharides, starch) and sucrose within the upper alimentary canal. The vast bulk of dietary polysaccharides are impervious to human digestion and they transit to the colon where they are depolymerized and fermented into short chain fatty acids by the DGM, which are subsequently utilized by the host. One of the hallmark features of the DGM is the prevalence of bacteria such as Bacteroides spp whose genomes encode a large number of carbohydrate active enzymes (CAZymes) dedicated to the metabolism of dietary fiber. The colon is a highly competitive microbial ecosystem with diverse species competing for limited nutrients. In order to persist, intestinal residents occupy select niches or adapt successful metabolic strategies. One such strategy has been exploited by the Bacteroidetes, which has many members that operate as nutritional ‘generalists’. Generalists harness catabolic machinery targeting a wide variety of complex carbohydrates, the major nutrient available to the DGM. Thus the extensive repertoire of CAZymes enable the organisms of the DGM to respond to the variation in available dietary nutrients. To optimize efficiency and limit metabolic cost, Bacteroides spp. organize their carbohydrate metabolic pathways into Polysaccharide Utilization Loci (PULs) which are independently regulated functional units. Each targets a specific glycan. SusC/D-like proteins (named after the starch utilization system, the first characterized glycan degrading system in Bacteroides) function as outer-membrane bound protein complexes that recruit carbohydrates at the cell surface and facilitate transport in a predicted TonB activated process. Other features of PULs include regulatory proteins that sense the presence of a targeted substrate and activate expression of a pathway consisting of depolymerising enzymes, which release oligosaccharides and monosaccharides from polysaccharide substrates. Investigating the molecular basis of how CAZymes from Bacteroides spp dismantle structurally complex dietary carbohydrates has become a highly-successful strategy for enzyme discovery.

Similar to the metabolism of dietary glycans such as fructans and xylan and xylglucan, B. theta contains PULs that are dedicated for yeast mannann metabolism; however, it is a more complex process and represents an exception to the ‘one PUL for one substrate’ paradigm. Three PULs (MAN-PUL1/2/3) are induced by yeast mannann, and a fourth PUL (HMNG-PUL) that targets structurally related high mannose N-glycans is induced by Man₈GlcNAc₂. Using a series of biochemical approaches and targeted gene disruption, we were able to define the function of the majority of genes products encoded within MAN-PUL1/2/3 (Table 1). These enzymes work interdependently to completely saccharify yeast mannann in a process that initiates on the cell surface and culminates with intracellular mannose. Intriguingly, MAN-PUL1 and MAN-PUL2 display a high level of synteny; however, only MAN-PUL2 is...
indispensable, suggesting that there is functional specialization that has co-evolved within these pathways. This is exemplified by the capacity of the mannan PULs to accommodate diverse sugars and/or linkages found in other fungal species, such as Candida albicans and/or linkages found in other fungal nan PULs to accommodate diverse sugars exemplified by the capacity of the mannooligosaccharide metabolism by Schizosaccharomyces pombe. These findings suggest that high-mannose containing complex carbohydrates are valuable nutrients for B. theta proliferation or it exposes an important role for the symbiotic turnover of fungal carbohydrates in the intestine; 2 roles that may not be mutually exclusive. Indeed, there is a strong selective pressure for yeast mannan degradation and its significance for host health may extend beyond nutrient acquisition. Thus, while MAN-PULs are activated in the mammalian host, deletion of these genetic loci confers a significant advantage over the wild type bacteria in diets lacking the yeast glycan, and this metabolic trait is very highly conserved in different strains of B. theta.

**Mannan utilization is a selfish process**

Depolymerisation of yeast mannan is metabolically expensive. The complex, highly branched substrate greatly restricts enzyme access and its degradation requires a large number of enzymes (Table 1). In the metabolism of other complex carbohydrates, some organisms simplify transport by centralizing depolymerisation outside of the cell. For example, the ‘cellulosome’ is a large multienzyme complex found within some members of Clostridia that often tethers an extensive repertoire of plant cell wall degrading glycanases on the cell surface (Fig. 2). Extracellular processing comes at cost, however, as simple sugars released from complex substrates by ‘keystone species’ become accessible to other local residents. In these scenarios, carbohydrate metabolism is a ‘shared’ process and individuals can occupy ecological niches along catabolic cascades by specializing in stratified levels of carbohydrate or metabolite utilization. The model of yeast mannan metabolism by B. theta represents a ‘selfish’ alternative to this theme as limited processing occurs outside of the cell (Fig. 2). Complex mannooligosaccharides are generated by first exposing the α-1,6 mannan backbone at sparse sites and then shuffling these branched fragments across the outer membrane. Importantly, the extracellular enzymes that cleave the backbone operate at noticeably slower rates than their counterparts within the periplasm. This ensures that fragments are produced at a rate that will not saturate the transport equilibrium, which would result in loss of excess products to the environment.

Co-culturing experiments with B. theta and Bacteroides xylanisolvens and Bacteroides cellulosilyticus, 2 species that cannot metabolize yeast mannan but can grow on mannosse, support this selfish model. When provided with only yeast mannan as a carbon source, B. theta growth rates are unperturbed in co-culture whereas both of its relatives do not exhibit significant growth. This finding underpins that

**Table 1. MAN-PUL1/2/3 gene products involved in yeast mannan metabolism**

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<td>BT3862</td>
<td>GH99</td>
<td>E</td>
<td>BT3863</td>
<td>SGBP</td>
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1Locations are predicted using the LipoP 1.0 tool or inferred using biochemical data. C = cytoplasm; IM = inner membrane; P = periplasm; OM = outer membrane; E = extracellular surface.
2Activities have been validated experimentally.
3Reported in. SGBP = surface glycan binding protein.
B. theta deploys a selfish mode of yeast mannan metabolism and prevents the superfluous release of mannose or mannooligosaccharides into the media to be shared with other species. Significantly, this relationship may be substrate specific, and one needs to be cautious before extrapolating whether B. theta is solely selfish in its metabolism, as a selective sharing relationship was recently demonstrated between Bacteroides ovatus and Bifidobacterium adolescentis, and several members of Bacteroides with differing growth aptitudes were able to liberate oligosaccharides (i.e. “public goods”) from multiple carbohydrate sources into solution for utilization by other species. The tendency of PULs to concentrate complex oligosaccharides depolymerising enzymes within the periplasm, however, suggests that B. theta is primarily concerned with self-nourishment and symbioses with other species may be dependent on the chemistry or complexity of the substrate.

A coupled catabolic-biosynthetic cascade to reprofile exogenous mannos? MAN-PUL2 contains the first characterized example of coupled mannan catabolism and mannooligosaccharide biosynthesis in nature. A putative biosynthetic cassette contains 2 intracellular family 32 glycosyl transferases (GT32s; BT3775 and BT3776) that sequentially catalyze the synthesis of a branched α-mannose trisaccharide that is likely decorated further by other biosynthetic components encoded within MAN-PUL2 or inherent capsular polysaccharide synthesis pathways. To ensure that nascent α-Man₃ products are not indiscriminately digested the catabolic and anabolic stages of mannose processing are compartmentalized into the periplasm and cytoplasm, respectively.

Although the downstream role of α-Man₃ remains to be resolved, it is likely either a component of a storage molecule or a capsular polysaccharide. The typansomatid protozoan Leishmania spp. synthesizes an intracellular β-1,2 mannan that is harvested when glucose becomes limiting, and bacterial glycogen (i.e., α-1,4 (α-1,6)-glucan) is a storage reserve in over 40 different bacterial species. Alternatively, α-Man₃ may be incorporated into the capsule of B. theta. A previous study determined that disrupting Bt3775 or genes encoding the capsular polysaccharide 4 (CPS4) biosynthetic pathway removed a surface epitope recognized by 225.4, an IgA monoclonal antibody raised in a germ-free mouse colonized with B. theta. The potential interplay between yeast mannan metabolism, α-mannooligosaccharide synthesis, and capsular remodeling represents a unique response of B. theta to dietary yeast and warrants further investigation.

Fungal immune perception

Chitin (β-1,4-N-acetylglucosamine) and β-1,3(β-1,6)-glucans are the primary structural polysaccharides of the fungal cell wall. These repetitive structures are not found within human glycans, and therefore, make ideal targets for the recognition of foreign symbionts or opportunistic pathogens (Fig. 1). In this regard, the perception of fungal polysaccharides has been linked to numerous innate and adaptive immune responses involving surveillance proteins, such as dectin-1 (Dec-1), mannos receptor (MR), and toll-like receptor 2 (TLR2). Yeast mannan on the other hand shares compositional and stereochemical similarity with human high mannose N-glycans; although, it is substantially larger and its sidechains are capped with Man-α-1,3-Man instead of Man-α-1,2-Man (Fig. 1). In addition, carbohydrate heterogeneity within fungal

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**Figure 2. Strategies for extracellular digestion of complex carbohydrates.** (A) The ‘sharing’-strategy: extracellular enzymes or enzyme complexes (e.g., Cellulosomes) process complex carbohydrate substrates into simple sugars and small oligosaccharides. These substrates can be readily utilized by any proximal bacterium. In Gram (+) bacteria products are bound by solute binding proteins (SBPs) and transported across the cell wall by ATP-dependent transporters MNA₂. (B) The ‘selfish’-strategy: minimal extracellular processing of the substrate. Complex oligosaccharides are transported into the periplasm through the Sus-like transport system where the bulk of depolymerisation occurs. Monosaccharides enter the cytoplasm through a major facilitator superfamily (MFS) transporter. Variations of this pathway exist as complex oligosaccharides released during extracellular processing may be selectively used by other bacteria in the ecosystem (indicated with a yellow star); however, they remain inaccessible to the majority of bacteria within proximity.
mannans, such as the β1,2 mannosyl caps that decorate the surface of the opportunistic pathogen *Candida albicans*, are structural signatures that can be detected by other immune sentinel proteins, such as the C-type lectin receptor dectin-2 (Dec-2); mannose binding lectin (MBL),28 and MR29 and dendritic cell receptor (DC-SIGN),30 a process which can involve combinatorial interactions (Fig. 1).

The sidechains of yeast mannan have been linked to perturbed immune responses in patients with Crohn’s disease (CD), a debilitating inflammatory autoimmune disorder that presents in the small bowel and colon. Anti-S. cerevisiae antibodies (ASCA) preferentially recognize the sidechain structure of yeast mannan (Man-α-1,3→Man-α-1,2→Man),31 and have been found in higher titres in the sera of CD patients. Intriguingly, the DGM of CD patients has a marked reduction in the abundance of *B. theta* in comparison with healthy individuals (~35%).32 Additionally, higher levels of *Bacteroides* spp have been associated with CD patients in remission when compared to those that have relapsed.33 It is tempting to speculate that this relationship may result, at least in part, from a reduced ability of a compromised DGM (i.e. lacking *B. theta*) to tolerate and digest mannans from dietary or endogenous yeasts.

In culture *B. theta* PUL-MAN expression is high within its carbohydrate metabolism hierarchy, and these pathways are actively expressed *in vivo*, underpinning the importance of yeast mannan responsive enzymes. For example, BT3862 is an extracellular endo-α-1,2-mannanase from MAN-PUL3 that prunes yeast mannan sidechains by specifically cleaving within the ASCA epitope and releasing Man-α-1,2→Man2. Exploiting this relationship may have therapeutic potential for alleviating ASCA-associated exacerbations and *B. theta* has received orphan drug status by the FDA for the treatment of pediatric CD. In this light, harnessing the mechanistic roles of naturally occurring members of the DGM for improving intestinal health and developing applications for offsetting dysbioses that result from improper regulation of DGM community structure is a promising research avenue for next generation ‘live-culture’ biologics.

**Conclusion**

The analysis of yeast mannan degradation by *B. theta* provides a model for the depolymerisation and utilization of complex sterically constrained carbohydrates by *Bacteroides*. The bacterium has evolved a surface enzyme system that is optimized to produce a large number of oligosaccharides for import into the periplasm where depolymerisation is completed. This mechanism minimizes the energy used in the import process and the loss of nutrients to the environment, and thus represents a selective advantage for the bacterium. While this cellular deployment pattern of enzyme systems is conserved for other glycanas, the discriminating factor between selfish and sharing metabolism appears to be substrate dependent. Extracellular digestion of ‘accessible’ glycans (i.e., sterically unconstrained) results in the release of oligosaccharides into the environment where they become available to other organisms in the DGM.16,21 Both the sources of yeast mannan molecules and the significance of their turnover by the DGM for host health may extend beyond nutrient acquisition. For example, recent efforts have been aimed at defining and enumerating the fungal members of the microbiota ("mycobiome") during health and disease and it is possible that *B. theta* has evolved to utilize mannans from a variety of endogenous yeasts that colonize the gut. In addition, while cultural consumption of foods that are fermented with yeast is a uniquely human endeavor, yeasts are naturally present in a variety of foods such as ripe and spoiled fruits and may therefore have impacted microbiota evolution in other animals as well. Regardless of the source, it should be emphasized that there is apparently a strong selective pressure for yeast mannan degradation in *B. theta*. This evidence emanates from our observation that these 3 PULs are consistently activated in the mammalian host even in the absence of usable mannan, perhaps in response to an inducing signal derived from endogenous N-glycans or another source that mimics the presence of the yeast polysaccharide. Surprisingly, deletion of these loci in *B. theta* conveys a substantial competitive advantage over the wild type bacterium in diets lacking the yeast glycana, while wild-type bacteria compete best when bona fide mannan is present as a nutrient. Thus, while deployment of this ‘expensive’ metabolic trait decreases the fitness of *B. theta*, it has remained highly present in nearly all strains of this species that were analyzed.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Rhizobial and Mycorrhizal Symbioses in *Lotus japonicas* Require Lectin Nucleotide Phosphohydrolase, Which Acts Upstream of Calcium Signaling
Rhizobial and Mycorrhizal Symbioses in Lotus japonicus Require Lectin Nucleotide Phosphohydrolase, Which Acts Upstream of Calcium Signaling1[C][W][OA]

Nicholas J. Roberts2,3, Giulia Morieri2, Gurpreet Kalsi, Alan Rose, Jiri Stiller, Anne Edwards, Fang Xie, Peter M. Gresshoff, Giles E.D. Oldroyd, J. Allan Downie, and Marilynn E. Etzler*

Department of Molecular and Cellular Biology, University of California, Davis, California 95616 (N.J.R., G.K., A.R., M.E.E.); John Innes Centre, Norwich NR4 7UH, United Kingdom (G.M., A.E., F.X., G.E.D.O., J.A.D.); and Australian Research Council Centre of Excellence for Integrative Legume Research, University of Queensland, Brisbane, Queensland 4072, Australia (J.S., P.M.G.)

Nodulation in legumes requires the recognition of rhizobially made Nod factors. Genetic studies have revealed that the perception of Nod factors involves LysM domain receptor-like kinases, while biochemical approaches have identified LECTIN NUCLEOTIDE PHOSPHOHYDROLASE (LNP) as a Nod factor-binding protein. Here, we show that antisense inhibition of LNP blocks nodulation in Lotus japonicus. This absence of nodulation was due to a defect in Nod factor signaling based on the observations that the early nodulation gene NODULE INCEPTION was not induced and that both Nod factor-induced perinuclear calcium spiking and calcium influx at the root hair tip were blocked. However, Nod factor did induce root hair deformation in the LNP antisense lines. LNP is also required for infection by the mycorrhizal fungus Glomus intraradices, suggesting that LNP plays a role in the common nodulation pathway shared by the rhizobial and mycorrhizal symbioses. Taken together, these observations indicate that LNP acts at a novel position in the early stages of symbiosis signaling. We propose that LNP functions at the earliest stage of the common nodulation and mycorrhization symbiosis signaling pathway downstream of the Nod factor receptors; it may act either by influencing signaling via changes in external nucleotides or in conjunction with the LysM receptor-like kinases for recognition of Nod factor.

The acquisition of mineral nutrients from the environment often limits plant growth, and many plants have established symbiotic interactions with beneficial microorganisms that facilitate nutrient acquisition. The association with arbuscular mycorrhizal fungi helps in nutrient uptake, particularly phosphate, and this symbiosis is almost ubiquitous within the plant kingdom, reflecting its early establishment during the evolution of higher plants (Parniske, 2008). Several plant species also form symbioses with nitrogen-fixing bacteria such as rhizobia and Frankia spp., and these symbioses are restricted to plants in the Rosid I clade (Doyle, 1998). The establishment of both mycorrhizal and rhizobial interactions in legumes involves a molecular signal exchange between the plant and its symbiont. Legumes release strigolactones and flavonoids into the rhizosphere, and these are recognized by mycorrhizal fungi and rhizobia, respectively (Oldroyd et al., 2009). In turn, the symbionts release signals to the plant: lipochitooligosaccharide Nod factors from rhizobia (Dénarié et al., 1996) and Myc factors from mycorrhizal fungi (Kosuta et al., 2003; Oláh et al., 2005; Maillet et al., 2011). Species-specific decorations on the Nod factor backbone are recognized by the host legume, and this determines specificity in the legume-rhizobial interaction (Perret et al., 2000). In contrast, there appears to be little specificity in mycorrhizal interactions (Parniske, 2008).

Nodulation-defective legume mutants have revealed a signaling pathway that is conserved between Nod factor and mycorrhizal recognition (Wais et al., 2000; Walker et al., 2000; Kistner et al., 2005), and considering the different evolutionary histories of these two
symbioses, it is legitimate to surmise that the evolution of nodulation involved the recruitment of the preexisting mycorrhizal signaling pathway for the recognition of Nod factor. At the core of this common symbiosis signaling (SYM) pathway are oscillations in nucleus-associated cytosolic calcium levels, termed calcium spiking, acting as a secondary messenger to induce gene expression. A receptor-like kinase (SYM/RK/DM12 [Endre et al., 2002; Stracke et al., 2002]), three components of the nuclear pore (NUP133, NUP85, and NENA [Kanamori et al., 2006; Saito et al., 2007; Groth et al., 2010]), and nucleo-localized cation channels (POLLUX/DM1, CASTOR [Ané et al., 2004; Imaizumi-Anraku et al., 2005; Charpentier et al., 2008]) are all required for the induction of calcium spiking, while a calcium-and calmodulin-dependent protein kinase (CCaMK [Lévy et al., 2004; Mítra et al., 2004; Tirichine et al., 2006]) and a protein of unknown function (CYCLOPS/IPD3 [Messinese et al., 2007; Yano et al., 2008]) are necessary for perception of the calcium signal and the induction of downstream genes. In addition to the common symbiosis pathway, Nod factor signaling also involves the nodulation-specific receptor-like kinases NFR1/LYK3 and NFR5/NFP (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006) that appear to function in Nod factor perception. There are also several nodulation-specific transcription factors that act downstream of CCaMK (Libault et al., 2009).

Genetic evidence indicates that different Nod factors are differentially perceived during Nod factor signaling (Ardourel et al., 1994), with perinuclear calcium spiking associated with early gene induction (Oldroyd and Downie, 2006) and calcium influx at the root hair tip proposed to be associated with the initiation of infection (Miwa et al., 2006a). This suggests a highly specific Nod factor recognition mechanism, which can discriminate between different Nod factor structures. The LysM receptor-like kinases NFR1/LYK3 and NFR5/NFP have emerged as strong candidates to function as Nod factor receptors based on the findings that purified NFR1 and NFR5 bind Nod factors with high affinity (Broghammer et al., 2012) and that the specificity of Nod factor recognition resides within the LysM domains (Radutoiu et al., 2007; Bek et al., 2010).

Whereas the LysM receptor-like kinases were identified using nodulation-defective mutants, we isolated a Nod factor-binding lectin from the legume *Dolichos biflorus* (also known as *Vigna unguiculata*; Quinn and Etzler, 1987). This protein can hydrolyze phosphoanhydride bonds of nucleoside triphosphates and diphosphates (Etzler et al., 1999). In mammalian systems, such enzymatic activity was proposed to remove extracellular ATP, known to be toxic to cells. This LECTIN NUCLEOTIDE PHOSPHOHYDROLASE (LNP) is a peripheral membrane protein present on the surface of epidermal root hairs in the zone of the root with the capacity to nodulate and is redistributed to the tips of the root hairs upon exposure to rhizobia or Nod factors (Kalsi and Etzler, 2000). The importance of LNP for the establishment of the rhizobial symbiosis was indicated following pretreatment of the roots with antiserum against recombinant LNP that inhibited both root hair deformation and nodulation in response to rhizobia (Etzler et al., 1999; Day et al., 2000). Two soybean (*Glycine max*) apyrases have been characterized, the Golgi-localized endoapryrase GS50 and the plasma membrane-associated ectoapryrase GS52 (Day et al., 2000). GS52 is rapidly induced by rhizobial inoculation (Day et al., 2000), its overexpression increased both nodulation and rhizobial infection (McAlvin and Stacey 2005), and the apyrase activity was required for the enhanced nodulation (Tanaka et al., 2011). Furthermore, silencing of GS52 expression by RNA interference (RNAi) reduced nodule development and infection (Govindaraju et al., 2009); this phenotype was partially rescued by the addition of ADP, implying that a role for the ectoapryrase may be to produce ADP from the extracellular ATP that is present around root hair tips (Kim et al., 2006). These data suggest that the role for the apyrase activity could be in the production of ADP rather than the reduction of ATP levels. The roles of apyrases and extracellular nucleotides in plant signaling have been reviewed recently (Clark and Roux, 2011).

These studies indicate that LNP binds Nod factor and is necessary for nodulation signaling. In parallel, genetic studies implicate the LysM receptor-like kinases in specific Nod factor recognition (Radutoiu et al., 2007), and high-affinity Nod factor binding to purified NFR1 and NFR5 has been demonstrated recently (Broghammer et al., 2012). In order to better understand the function that Nod factor binding to LNP plays in the establishment of symbiotic interactions, we generated transgenic lines of *Lotus japonicus* that have markedly reduced LNP levels. These lines are defective for both nodulation and mycorrhization, suggesting that LNP plays a role in the common symbiosis signaling pathway. Nod factor-induced responses such as gene induction, calcium influx, and calcium spiking were absent, but root hair deformation was retained, implying that LNP functions at a very early stage in the symbiosis signaling pathway.

**RESULTS**

**Isolation of Stable Antisense Transformants Lacking LNP**

*L. japonicus*, ecotype Gifu, was stably transformed with three different *L. japonicus* LNP antisense constructs under the control of the constitutive 35S promoter. The full-length antisense construct contained the complete 1,489-nucleotide complementary DNA from *L. japonicus* (Roberts et al., 1999), whereas the 5′ and 3′ constructs contained nucleotides 1 to 719 and nucleotides 536 to 1,383, respectively. Seven independent transformed lines (one with the full-length construct, three with the 3′ construct, and three with the 5′ construct) were selected from independent calli based on their strong kanamycin resistance. Extracts of the uninoculated roots from the seven lines, the wild type, and the vector control were immunoblotted...
with antiserum to LNP. Three of the transformed antisense lines, named 5’D, 5’R, and 3’O (generated using the 5’ or 3’ construct), had substantially reduced levels of immunoreactive material compared with the wild type (Fig. 1A), so these lines along with the vector controls were propagated for seed production. The initial T0 transformed plants were selfed, and T1 seed were selected on antibiotic plates. Homozygous T1 lines were identified by testing their progeny for both kanamycin resistance (imparted by the transformed vector) and LNP protein levels using immunoblotting. If all the tested progeny from a single line were kanamycin resistant and showed substantially reduced LNP levels, we considered these lines to be homozygous. This approach was taken for 5’D, 5’R, 3’O, and the vector control lines, and homozygosity was achieved for each construct in different generations (T1, T2 or T3), and thus T2, T3, or T4 seed was used accordingly for further analysis.

LNP can be detected around the surface of root hairs using immunofluorescence (Kalsi and Etzler, 2000; Fig. 1E). To validate the LNP suppression in the 5’D, 5’R, and 3’O lines, we assessed the protein levels on the surface of the root. Whereas the wild type and the vector control lines showed good levels of LNP on the root surface (Fig. 1, D–G), the 5’D, 5’R, and 3’O lines showed no detectable levels of LNP (Fig. 1, H–M).

Figure 1. Immunoblot and confocal immunofluorescence microscopic analyses of LNP from wild-type (WT), vector control, and transgenic antisense lines. A, Equivalent amounts of extract from pooled roots of uninoculated 8-d-old L. japonicus lines (Table I) were subjected to SDS-urea-PAGE followed by immunoblot analysis using antiserum prepared against recombinant LNP. The single immunoreactive band corresponds to approximately 46 kD, the predicted size of APY1. B–M, Confocal immunofluorescence microscopy was performed using preimmunization serum (B and C) or anti-LNP serum (D–M) on whole mounts of fixed 7-d-old uninoculated roots from the following L. japonicus lines: the wild type (B–E), vector control (F and G), 5’D (H and I), 5’R (J and K), and 3’O (L and M). Bars = 50 μm.

Searching the genome sequence of L. japonicus (Sato et al., 2008) revealed a second gene (chr1.CM0104.1830. r2.d) with clear similarity to LNP; the DNA and predicted protein sequences of the coding regions of this gene are 58% and 72% identical. We have called this gene LNP2, and to understand how these two genes are related to each other and to other apyrases, we generated a phylogenetic comparison of 68 plant apyrases and integrated this with an in silico prediction of their targeting sequences (Supplemental Fig. S1). From this combined analysis, there are a number of observations that can be made. Most of the sequences were predicted to contain one of three types of putative targeting sequence (secretory pathway, mitochondrial, or chloroplast). Among the proteins with predicted secretory targeting sequences, there were three clades, which could be separated into Fabaceae (including DbLNP, LjLNP, and GS52, which appear to be orthologs based on the phylogenetic analysis), Poaceae, and Solanaceae, with representatives from Brassicaceae. Within the Fabaceae clade, there are considerably more apyrases among those legumes (e.g. pea [Pisum sativum] and Medicago truncatula; Cohn et al., 2001; Navarro-Gochicoa et al., 2003) that form indeterminate nodules than those (e.g. Glycine soja and L. japonicus) that form determinate nodules.

D. biflorus apyrase2 and soybean GS50 form a separate grouping within the predicted ectoapyrases. LjLNP2 falls within the clade of legume apyrases that are predicted to contain mitochondrial targeting sequences and is related to the Arabidopsis (Arabidopsis thaliana) apyrases APY1 and APY2, which appear to be involved in regulating stomatal aperture (Clark et al., 2011); the double apy1, apy2 mutant is male sterile (Steinebrunner et al., 2003).

We used quantitative reverse transcription (RT)-PCR with gene-specific primers to assay the levels of transcripts of the two LjLNP genes in the 5’D LNP antisense line and the empty vector control. We found an average 53% ± 22% reduction in the transcript levels of the LNP gene to which we made the construct and 46.5% ± 28% reduction in LNP2 transcript levels in the 5’D antisense line (from four independent biological replicates). Antisense is likely to function via both transcript stability in an RNAI-type mechanism and possibly through suppression of translation. Hence, the absolute levels of transcript abundance are unlikely to be the only cause of the dramatic reductions we observed in the level of LNP protein in roots. These results indicate that the LNP antisense construct may target both LNP genes, and this may explain why we observed relatively poor seed set and seed viability with some antisense lines. The two LNP genes are located in very close proximity (separated by only about 30 kb) on the genome, and this greatly limits the ability to generate a double mutant line. However, the key observation is that LNP is not antigenically detectable on the root surface and, therefore, is not available to function in symbiosis.

Antisense Suppression of LNP Results in a Nod” Phenotype

The homozygous 5’R (T2), 5’D (T3), and 3’O (T4) lines were tested for their ability to nodulate by
inoculating 10-d-old seedlings with *Mesorhizobium loti* strain NZP2235. No nodules formed on the 5’D, 5’R, or 3’O plants (Table I); in comparison, the wild type and the vector control plants formed nodules (Table I; Fig. 2). When grown in the absence of added nitrate or ammonia, the nodulated plants all grew more vigorously than the nonnodulated plants (Table I), confirming that the nodules were fixing nitrogen. The fact that the three independent LNP knockdown lines were all defective for nodulation demonstrates that the loss of LNP caused the nodulation defect.

### Antisense Suppression of LNP Inhibits the Ability of Plants to Associate with Mycorrhizal Fungi

Many mutants defective for early nodulation signaling also fail to form symbioses with arbuscular mycorrhizal fungi (Parniske, 2008), and mycorrhizal fungi induce calcium spiking during initiation of the symbiosis (Kosuta et al., 2008). Mutations in NFR1 or NFR5 do not block mycorrhization, but mutations in genes of the SYM pathway do block the normal infection by mycorrhizal fungi (Parniske, 2008). We investigated the ability of the three LNP antisense lines, 5’D (T3), 5’R (T2), and 3’O (T4), to be colonized by *Glomus intraradices*. Ten plants were assessed at each time point, and roots were stained and analyzed for fungal colonization, with a total of 50 plants analyzed for each line. Nine days post inoculation (dpi), the attachment of fungal hyphae and appressoria formation were observed on the epidermal cell surface of all roots, and no difference was observed between the wild type, the vector control, and the three LNP antisense lines (Fig. 3, A–E). At 15 dpi, roots of both wild-type and vector control plants were heavily colonized by *G. intraradices*, with fungal colonization of inner cortical cells forming arbuscules (Fig. 3, F and G). No such invasion of roots by fungal hyphae was observed in the 5’D, 5’R, and 3’O lines (Fig. 3, H–J), and this lack of colonization was sustained even at 35 dpi, when wild-type and vector control roots were strongly colonized by *G. intraradices* (Fig. 3, K and L). Enhanced branching of fungal hyphae was observed on the epidermal cells of LNP antisense lines compared with the wild type and vector control, possibly because of the unsuccessful attempts of the fungal hyphae to gain entry into roots (Fig. 3, M–O). No balloon-like swellings of fungal hyphae or deformations were observed on the epidermal or inner cells of these three antisense lines at any time point. At 60 d after inoculation with *G. intraradices*, lines 5’D and 5’R showed some fungal colonization, but this was observed in only 6% of the root segments of the antisense lines compared with approximately 90% of the root segments obtained from the wild type. These results show that these three LNP antisense lines are defective for the initiation of the symbiotic association with mycorrhizal fungi, and this block is at the epidermal cell surface.

![Figure 2. Root nodule phenotype of the *L. japonicus* 5’D LNP antisense line. Nodules formed in wild-type plants (A and D) and vector control (B and E) 3 weeks after inoculation with *M. loti*. No nodules were observed in the 5’D LNP antisense line (C and F). Bars = 10 mm (A–C) and 5 mm (D–F). [See online article for color version of this figure.]](image)

| Table 1. Nodulation in LNP antisense lines 4 weeks post inoculation with *M. loti* |
|---------------------------------|------------------------|-----------------|-----------------|
| **Line**                        | **Generation**        | **No. of**      | **Shoot Mass** |
|                                 | **Shoot Mass**        | **Plant**       | **Root Mass**   |
| Wild type                       |                       | 23.7 ± 3.2      | 16.7 ± 4.4      | 10.2 ± 2.7      |
| pHIn19                          | T3                    | 13.3 ± 2.4      | 13.0 ± 1.4      | 8.0 ± 0.4       |
| 5’D                             | T3                    | 0.0 ± 0.0       | 8.3 ± 0.2       | 6.0 ± 0.7       |
| 5’R                             | T2                    | 0.0 ± 0.0       | 5.9 ± 1.3       | 6.1 ± 0.2       |
| 3’O                             | T4                    | 0.0 ± 0.0       | 4.4 ± 0.7       | 4.8 ± 0.7       |

LNP Is Required for Nod Factor Induction of NODULE INCEPTION Expression

All three LNP antisense lines showed defects in rhizobial and mycorrhizal colonization, implying that LNP acts in the common symbiosis signaling pathway. To test this, we assessed features of symbiosis signaling, using gene induction and calcium responses to Nod factor as the measure. The fact that all three independent antisense lines showed defects in symbiosis (Table I; Fig. 3) strongly supports the fact that this must be the result of the suppression of LNP. From our observations, it appears that suppression of LNP may affect seed production and long-term viability of the
seed, since both seed yield and seed germination levels for these lines appeared to be reduced, and long-term storage was affected. The 5°D line was frequently propagated, but the other two lines were not, and the seed of the 5°R and 3°O lines were no longer viable when it came to the analysis of the SYM pathway. Thus, it has only been possible to measure SYM pathway activities in the 5°D line.

As an initial indication of the SYM pathway activity in the 5°D line, we assayed the transcription of NODULE INCEPTION (NIN), which is normally induced in L. japonicus a few hours after Nod factor treatment. The transcript abundance of this gene represents a useful indicator for the activity of Nod factor signal transduction, because mutations that block the Nod factor-induced signaling pathway in L. japonicus block NIN induction (Radutoiu et al., 2003; Imaizumi-Anraku et al., 2005; Miwa et al., 2006b; Marsh et al., 2007). NIN induction was observed in roots of wild-type plants and in the vector control line 24 and 48 h after Nod factor treatment (Fig. 4). In contrast, no significant induction of NIN transcript was seen in the 5°D LNP antisense line at any of the time points tested (Fig. 4). These data indicate that LNP is required for the full transcriptional activation of NIN and suggest that LNP functions in Nod factor signal transduction.

LNP Is Required for Nod Factor-Induced Calcium Responses

To test if LNP is required for Nod factor signaling, we measured Nod factor-induced changes in root hair calcium by dual-dye pseudoratiometric imaging following microinjection of the calcium-responsive dye Oregon Green and the reference dye Texas Red. The 5°D line was completely defective for Nod factor-induced calcium spiking; of 43 root hairs tested on 13 plants, none induced calcium spiking in response to Nod factor. In contrast, wild-type plants and the transgenic line carrying the empty vector were clearly positive for Nod factor-induced calcium spiking (Fig. 5). These data show that LNP, like NFR1, NFR5, and components of the common symbiosis signaling pathway, is required for Nod factor-induced calcium spiking.
LNP could act as part of a Nod factor receptor (e.g. together with NFR1 and NFR5) or could be required for the common symbiosis signaling pathway. A component that discriminates between these two scenarios is a root hair tip-focused influx of calcium induced by Nod factor that appears to be independent of calcium spiking (Miwa et al., 2006a). The nfr1 and nfr5 mutants are completely defective for both Nod factor-induced calcium influx and spiking, whereas symRK, nup133, castor, and pollux mutants are defective for Nod factor-induced calcium spiking but retain the calcium flux (Miwa et al., 2006a). As the calcium influx response is localized in the tip of the root hair (Shaw and Long, 2003), we analyzed changes in intracellular calcium in the cells and in the tip of every root hair tested; only those cells that induced a significant transient increase in tip calcium compared with other cytoplasmic or nuclear regions were considered positive for the calcium influx. We observed no significant Nod factor-induced changes in cytoplasmic calcium concentration in the 5’D line (n = 26 cells from 10 plants), whereas a clear response was seen with wild-type plants and the empty vector control plants (Fig. 6). These results provide evidence that LNP is required for both Nod factor-induced calcium responses, as has been observed in nfr1 and nfr5 mutants (Miwa et al., 2006a).

To test the possibility that the generation of the 5’D antisense line might have caused silencing of NFR1 or NFR5 and that this could explain the observed phenotypes, we checked the expression of NFR1 and NFR5. Quantitative PCR analysis of the expression of NFR1 indicated that its expression, if anything, may be slightly increased in the 5’D antisense line (1.8-fold higher than in the vector control line), but the difference was not significantly different. NFR5 expression was also slightly increased in the 5’D antisense line (1.5-fold more than in the vector control), but again the difference was not significant. Based on these observations, we conclude that the phenotype of the 5’D antisense line is not due to an off-target effect causing silencing of NFR1 or NFR5. Since Nod factor-induced calcium influx is blocked in the 5’D antisense line, this phenotype cannot be explained by an off-target effect on any of the other known nodulation signaling genes.

LNP Is Required for Infection But Not for Root Hair Deformation

The finding that the knockdown of LNP resulted in the absence of calcium spiking, calcium influx, and NIN expression, together with the evidence showing that LNP binds Nod factor (Etzler et al., 1999), suggested that LNP could play a role in the perception of Nod factor. This would place LNP upstream of the common symbiosis signaling pathway in a position shared with NFR1 and NFR5. If LNP does indeed form a key component of Nod factor perception, the antisense line would be expected to lack all Nod factor responses. Root hair deformation is an early response to Nod factor, but it can occur in some L. japonicus mutants defective for Nod factor-induced calcium spiking (Miwa et al., 2006a). Using a root hair deformation assay with increased sensitivity (Miwa et al., 2006a), the only mutants that did not show root hair deformation responses to Nod factor were those lacking NFR1 or NFR5 (Radutoiu et al., 2003; Miwa et al., 2006a). Using this assay, the 5’D line clearly showed a significant root hair deformation (but not the shepherd’s crook structures typical of full root hair curling) in response to both M. loti inoculation and to 10 nM Nod factor (Fig. 7) and 100 nM Nod factor (P < 0.001).
This result, which is somewhat different from that described previously using a less sensitive assay (Etzler and Roberts 2005), reveals that the LNP antisense line is able to perceive and respond to Nod factor, suggesting that LNP probably acts downstream of Nod factor perception by NFR1 and NFR5 or in a parallel position to these receptor-like kinases.

In view of the fact that we observed root hair deformation, we scored seedlings for infection foci and infection threads per seedling and 6 weeks after inoculation of wild-type plants, we detected an average of 37 ± 12 (n = 24) infection threads per seedling and 6 ± 3 (n = 24) infection foci that had not progressed to infection threads. In the 5′D antisense line, no infection threads were observed, and we observed an average of 0.5 ± 1 (n = 22) potential infection foci. In other lines in which infection is blocked, the number of infection foci is greatly increased compared with the wild type (Xie et al., 2012), so the very few potential infection foci observed suggest that even the formation of infection foci is mostly blocked.

DISCUSSION

LNP was initially identified for its Nod factor-binding ability, with preferential binding to Nod factor relative to chitin (Etzler et al., 1999). The localization of LNP to legume root hair cells, its rhizobia-induced accumulation at root hair tips, the ability of LNP antiserum to interfere with nodulation (Etzler et al., 1999; Kalsi and Etzler, 2000), and the finding that silencing LNP in soybean reduces nodulation (Govindarajulu et al., 2009) all pointed to a function for LNP in nodulation signaling. In this work, we inhibited the formation of LNP and revealed that in three independent antisense lines, there was no nodulation following inoculation with M. loti. Furthermore, these three LNP antisense lines also showed defects in mycorrhizal colonization. Using one of the antisense lines, we showed that LNP is required for Nod factor-induced gene expression, calcium spiking, and calcium influx, implying a role for LNP in Nod factor signaling. Our work places LNP at a novel position, very early in the common symbiosis signaling pathway (Fig. 8).

Previously characterized components of symbiosis signaling fall into four major classes: the hypothetical Nod factor receptors NFR1 and NFR5/NFP are necessary for all Nod factor responses so far measured but are not required for mycorrhization (Madsen et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006); SYMRK/DMI2, CASTOR, POLLUX/DMI1, NUP133, NUP85, and NENA are necessary for Nod factor-induced calcium spiking and gene expression as well as mycorrhization but are not required for calcium influx or root hair deformation (Wais et al., 2000; Endre et al., 2002; Stracke et al., 2002; Kanamori et al., 2006; Miwa et al., 2006a; Saito et al., 2007; Groth et al., 2010); CCaMK and CYCLOPS have similar functions but sit downstream of calcium spiking (Wais et al., 2000; Miwa et al., 2006a; Yano et al., 2008); while NSP1, NSP2, ERN, and NIN are nodulation-specific transcription factors downstream of the SYM pathway (Stracke et al., 2002; Kaló et al., 2005; Smit et al., 2005; Marsh et al., 2007; Middleton et al., 2007). From this genetics perspective, LNP represents a novel class. Like NFR1 and NFR5/NFP, LNP is required for both calcium influx and calcium spiking, but unlike NFR1 and NFR5/NFP, LNP is not required for root hair deformation and is required for mycorrhization. Such a phenotype implies a function for LNP in the common SYM pathway, but unlike LNP, components of the common SYM pathway are not required for Nod factor-induced calcium influx. Thus, the phenotype of the LNP antisense lines suggests a novel position for LNP in symbiosis signaling lying between the LysM receptor-like kinases and the common SYM pathway (Fig. 8).

NFR1 and NFR5 are Nod factor receptors based on the observations that (1) they encode receptor-like kinases with LysM domains known to function in polysaccharide binding (Madsen et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006); (2) nfr1 and nfr5 mutants show no Nod factor responses (Radutoiu et al., 2003; Miwa et al., 2006a); (3) NFR1 and NFR5 bind Nod factors with high affinity (Broghammer et al., 2012); (4) the specificity of Nod factor recognition has been shown to be a function of the LysM domains of NFR5 (Bek et al., 2010); and (5) transfer of NFR1 and NFR5 to M. truncatula transferred the specificity of the L. japonicus rhizobial interaction (Radutoiu et al., 2007).
We considered the possibility that LNP might work in combination with the LysM receptor-like kinases during Nod factor perception. LNP could bind Nod factor and present it to the LysM receptor-like kinases and could even function as a complex with NFR1 and NFR5, possibly influencing their output. TFs, Transcription factors. The model is modified from that described previously (Miwa et al., 2006a); the ion channel genes are CASTOR and POLLUX, and the nucleoporin genes are NUP133, NUP98, and NENA, as described in the text. The solid lines indicate the proposed genetic pathway, and the broken lines show the induced phenotypes.

An alternative hypothesis is that LNP is a component of the common SYM pathway. Unlike LNP, the SYM pathway is not necessary for the Nod factor-induced calcium influx response (Miwa et al., 2006a), although it has been reported that M. truncatula mutated in dmi1 or dmi2 cannot support the complete calcium influx response (Shaw and Long, 2003). Furthermore, analysis in L. japonicus mutants of SYM pathway components identified various steps of mycorrhizal colonization of roots (Novero et al., 2002; Demchenko et al., 2004; Kistner et al., 2005). In all the early L. japonicus mutants defective for arbuscular mycorrhization identified so far, fungal hyphae have been observed to enter between epidermal cells, but the intracellular penetration of epidermal or outer cell layers is blocked except in Ljsym15, in which mycorrhization is blocked at the separation of anticlinal walls of epidermal cells and arbuscule formation (Demchenko et al., 2004). In the Ljsym15 mutant, fungal hyphae form appressoria and balloon-like deformations, but the subsequent fungal entry in cells of epidermis and exodermis was aborted, blocking the intracellular passage (Demchenko et al., 2004; Kistner et al., 2005). Attachment of fungal hyphae and appressorium formation were observed on the root surface of three LNP antisense lines, but intracellular or intracellular entry in the epidermal cells was not observed, implying a role for LNP in events leading to the epidermal cell entry of fungal hyphae. Thus, for both mycorrhizal signaling and Nod factor signaling, LNP appears to be acting upstream of other identified L. japonicus SYM genes. The predominant function of the SYM signaling pathway appears to be to transmit the recognition of symbiotic signaling molecules to the activation of calcium spiking and, ultimately, the transmission of this signal to transcription factors. It is difficult to ascertain the role for a second level of Nod factor binding that acts in SYM signaling but downstream of the LysM receptor-like kinases. However, one possibility is an interaction between LNP and SYMRK/DMI2, a receptor-like kinase whose function remains enigmatic.

A final scenario is one that places LNP in a parallel position to the LysM receptor-like kinases and the SYM pathway but possibly with a function required for the activation of symbiosis signaling (Fig. 8). Pertinent to this scenario is the apyrase activity of LNP. Presuming that the apyrase activity is relevant for Nod factor signaling, it is possible that either reducing the level of extracellular ATP or increasing the level of ADP or other degradation products of ATP may be important for symbiosis signaling. The mammalian extracellular apyrase NTDPase1/CD39 hydrolyzes extracellular ATP and/or ADP to AMP, which is subsequently degraded to adenosine. ATP, ADP, and adenosine serve as ligands to ligand-gated ion channel P2X receptors and G-protein-coupled P2Y and P1 receptors (Khakh and Burnstock, 2009). The addition of extracellular ADP increased legume nodulation and partially rescued the low nodule infection observed in soybean with RNAi of the expression of ectoapyrase GS52 (Govindarajulu et al., 2009), implying that ADP may be the key product of these apyrases. Alternatively, extracellular ATP may suppress symbiosis signaling. It has been suggested that extracellular ATP may act as a signal in plant-pathogen interactions (Demidchik et al., 2003), and pathogen-associated patterns such as chitin elicit increases in extracellular ATP (Kim et al., 2006). In theory, LNP could decrease ATP levels and thereby suppress plant defenses following recognition of the symbionts. However, such a role does not appear to fit well with the requirement for LNP in most aspects of symbiosis signaling. The effect of GS52 silencing in
soybean (Govindarajulu et al., 2009) primarily caused the formation of uninfected nodules, and this seems more likely to be due to an effect on infection; the observation that nodule primordia are formed suggests that in the GS52 RNAi line, activation of the calcium spiking pathway can occur because this is clearly linked to nodule morphogenesis (Oldroyd and Downie, 2006). However, it is possible that the calcium influx at the root hair tip could be affected, and this has been proposed to play a role in the initiation of infection (Miwa et al., 2006a). There are two ways in which LNP might influence infection rates. First, induction of the calcium influx requires a much higher level of Nod factor than does the induction of calcium spiking (Shaw and Long, 2003, Miwa et al., 2006a), so if GS52 silencing were to cause a change in sensitivity to Nod factor, it is possible that nodule morphogenesis could be induced but infection could mostly be blocked. Second, it is possible that Nod factor activation of LNP could increase extracellular ADP levels, and extracellular ADP has been shown to induce an inwardly directed calcium flux across the plasma membrane of root epidermal cells (Demidchik et al., 2011). This fits well with the proposal that a calcium influx may promote infection by rhizobia (Miwa et al., 2006a), but it does not fit so well with the observation that the soybean GS52 apyrase shows significantly higher activity with CTP, CDP, and ADP than it does with ATP (Tanaka et al., 2011).

If a major role for LNP is in infection, as appears to be the case with GS52 in soybean, it may be significant in that legumes (pea and M. truncatula) that form indeterminate nodules, there is much greater diversification of secreted apyrases (Supplemental Fig. S1), because in indeterminate nodules, infection threads are more widely distributed and more persistent. Possibly in legumes forming indeterminate nodules, there has been selection for multiple apyrases, and this redundancy could explain why no clear nodulation phenotypes have been observed in M. truncatula. The LjLNP antisense lines analyzed here have a more severe effect than RNAi of GS52 in soybean; the resulting block of nodule morphogenesis is consistent with the lack of Nod factor-induced calcium spiking.

Whatever the role for LNP, our work highlights a novel function for a Nod factor-binding protein in both mycorrhizal and rhizobial signaling. Considering the dual role for LNP in nodulation and mycorrhization, we can presume that LNP initially evolved as a function in mycorrhizal signaling that was secondarily recruited into nodulation signaling. In this regard, it may be significant that many plants, including Poaceae and members of the Solanaceae, have ectoapyrases, but no such ectoapyrases from Brassicaceae were found within this group. This correlates with the lack of infection of Brassicaceae plants by mycorrhizal fungi and may imply a role for these ectoapyrases in mycorrhization. The work described here reveals an additional parallel mechanism in the establishment of these two symbiotic processes, implying novel commonalities in proteins that bind the microbe-derived symbiotic signals.

MATERIALS AND METHODS

Biological Materials

_Lotus japonicus cv Cita_ seeds were gifts from Dr. J. Webb (Institute of Grassland and Environmental Research) and Dr. K. Szczygloowski (Michigan State University-Department of Energy Plant Research Laboratory). The seeds were scarified, surface sterilized, and germinated in Hoagland medium lacking nitrate and ammonium (Hoagland and Arnold, 1950). The seedlings were grown in petri dishes containing 0.7% to 1.0% phytagar (Gibco BRL) or filter paper imbedded with the above medium for 4 to 14 d before use. _Mesorhizobium loti_ and _Bradyrhizobium sp._ 24A10 (Lipha Tech) were grown in 0.5% glucose acid, 0.1% Glu, and 0.1% yeast extract (in mineral salts vitamin base) medium (Nieuwkoop et al., 1987) and 0.5% tryptone, 0.3% yeast extract, and 6 mmol CaCl₂ medium (Rosenberg et al., 1981), respectively. For gene expression, calcium, and root hair deformation analyses, seeds of _L. japonicus_ were scarified using sand paper, soaked in 0.1% sodium hypochlorite for 15 to 20 min, washed five times in sterile water, and imbibed for 1 to 2 h in sterile water. Imbibed seeds were grown for 2 d on water agar medium in the dark and placed upside down to allow the roots to grow vertically. Subsequently, seedlings with similar root lengths (0.5–2 cm) were selected and transferred to Fahraeus nitrogen-free plant agar medium (Fahraeus, 1957). Filter paper was placed between the agar and the roots to prevent the roots growing into the agar. The roots were then covered by another filter paper to keep them moist. The petri dishes were incubated in a vertical position in a controlled environment (20°C/15°C, day/night cycles of 18/6 h), and the region of the petri dish containing the roots was covered with black plastic.

Plant Transformation

The three different _LjLNP_ constructs described were cloned into the XhoI site of the shuttle vector pDF51 (Pietrzak et al., 1986) in the reverse orientation relative to the cauliflower mosaic virus 35S promoter and terminator. The antisense cassette was purifi ed from pDF51 after EcoRI digestion and ligated into the EcoRI site of the binary vector pBlN19, which utilizes nosP as a selectable kanamycin resistance marker under the control of the Nos promoter (Bevan, 1984). _Agrobacterium tumefaciens_ strain AGL1 (Lazo et al., 1991) was transformed with these antisense vectors and a pBIN19 vector control. The resulting strains were used to transform _L. japonicus_ hypocotyls as described previously (Stiller et al., 1997) but with the following modifications: (1) plants were grown on regeneration medium for only 4 to 5 d; (2) no geneticin was used once the calli were placed on shoot induction medium; and (3) full strength cefotaxime was used to control agrobacterial growth on all shoot and root media. On some lines, successful root initiation was only achieved using the higher auxin concentration described in the hairy root regeneration protocol of Stiller et al. (1997). Regenerated plants were grown under greenhouse conditions, and seeds were tested for geneticin resistance on phytagel (Sigma-Aldrich) plates containing 5 μg mL⁻¹ geneticin, 1% Suc, and 1× Gamborg’s B5 medium (Gamborg et al., 1968), pH 5.5.

Immoноassays

Recombinant _LjLNP_, representing residues 1 to 414 of the mature protein (Roberts et al., 1999), was expressed in _Escherichia coli_ using the pET28c expression vector (Novagen). The protein was extracted from isolated inclusion bodies, further purified by ion-exchange chromatography, and used as an immunogen for the production of anti-LNP serum. Both this antisera as well as an antisera previously prepared against recombinant _LNP_ from _Dolichos biflorus_ (Etzler et al., 1999) reacted with the 46-kDa LNP in immunooblots of _L. japonicus_ roots.

Protein was extracted from _L. japonicus_ roots, run in SDS-urea-PAGE, and subjected to immunoblot analysis as described previously (Kaláš and Etzler, 2000). Whole mounts of fixed _L. japonicus_ roots were fixed, treated for 20 min with preimmunization serum or antisera, washed, and then treated for 20 min with fluorescein-labeled goat anti-rabbit IgG (Sigma-Aldrich). After washing, the roots were examined with a Leica TCS NT confocal microscope (Leica, Wetzlar) using a 488-nm excitation laser line and a 520-nm barrier filter.
Nodulation Assays

Four days after germination, approximately 50 uniform seedlings from each transgenic line were transferred to pots of 10 × 10 × 8 cm containing sterile vermiculite and perlite (1:1, v/v) and placed in a growth chamber. The seedlings were grown for another 6 d and then thinned to approximately eight plants per pot to maintain uniformity. Four pots from each line were then inoculated with 50 mL per pot of a fresh overnight culture of M. loti, diluted to 10⁸ cells mL⁻¹ with Hoagland solution (Hoagland and Arnold, 1950) lacking nitrate and ammonium. Preliminary studies established that this dosage of rhizobia is in excess of what is required to get the maximum number of nodules per plant. An additional one pot per line was used as an uninoculated control. Three LNP lines (5'D, 5'R, and 5'O) were tested in similar nodulation assays two more times and then tested again using 10⁷ rhizobia mL⁻¹. The placement of each inoculated pot was randomized in the growth chamber, and the plants were grown using a 26°C, 16-h-light (350 μmol m⁻² s⁻¹)/22°C, 8-h-dark cycle. The plants were watered daily using sterile water that was supplemented weekly with sterile Hoagland solution (Hoagland and Arnold, 1950) lacking nitrate and ammonium. Four weeks after inoculation, the plants were gently removed from the soil and washed, and the number of visible nodules was counted on each plant. The shoot and root material in each pot were separated and dried at 65°C for 2 d prior to weighing. L. japonicus wild-type plants, vector control, and 5'D LNP antisense line were grown in poly-styrene K-resin pods (Phytatrey; Sigma-Aldrich) containing sterile clay granules (Seramis). Plants were grown in a growth chamber (20°C/15°C, day/night cycles of 18/6 h) and inoculated with M. loti (optical density = 0.05).

Expression Analysis

RNA was extracted from root tissue using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions followed by DNase treatment (Ambion). Complementary DNA was prepared from 1 μg of RNA using the SuperScript II first-strand synthesis system for RT-PCR (Life Technologies, Invitrogen) using oligo(dT) primers according to the manufacturer’s protocol. Quantitative RT-PCR was performed using the CFX96 Real-Time System (Bio-Rad) using SYBR Green Master Mix (Sigma-Aldrich). The reference dye Texas Red-dextran 10,000 MW (Molecular Probes) were micro-injected by iontophoresis into L. japonicus seedlings as described by Miwa et al. (2006a). Nod factor was added to 100 μL of liquid Fahraeus nitrogen-free plant agar medium in the chamber containing the seedling to give an estimated final concentration of 100 nM. Cells were imaged on an inverted epifluorescence microscope (model TE2000; Nikon) using a monochromator (model Optoscan; Cairn Research) to generate specific wavelengths of light. Images were captured with a CCD camera (model ORCA-ER) and fluorescence data were analyzed using MetaFlor (Molecular Devices). For calcium flux analysis, fluorescence imaged at the tip and at the shaft of the root hair were taken for each cell and compared with each other. The tip area included about 10 μm from the tip of the root hair, whereas the shaft area included part of the root hair cell protruding from the root. Only those cells showing a calcium increase in the area of the root hair cell protruding from the root as well as the root hair tip were considered positive for calcium flux. After taking a series of images, the pseudoratiometric traces were calculated by dividing Oregon Green fluorescence by that of Texas Red at each time point. Derivative traces represent the change in fluorescence intensity of Oregon Green/Texas Red from one point to the next (Δxᵣ/Δt). Traces were generated using Microsoft Excel.

Root Hair Deformation and Infection Assays

Seedlings prepared as described previously (Miwa et al., 2006a) were assayed for root hair deformation 24 h after the addition of 10 nM Nod factor or an inoculum of freshly grown M. loti at about 10⁷ cells mL⁻¹. Root hairs were examined by light microscopy, and root hair deformation was scored without prior knowledge of the treatment to the seedlings. The analysis included 25 untreated seedlings, 13 treated with 10 nM Nod factor, 23 treated with 100 nM Nod factor, and 13 inoculated with M. loti. Root hair deformation was quantitatively scored from 0 to 3, and the data were analyzed using Genstat version 13 using a two-way ANOVA. Images were taken using an inverted microscope with a digital camera. Infection threads were scored as described previously using M. loti R7A marked with lacZ and stained with 5-bromo-4-chloro-3-indoly-phosphate/sodium as described previously (Lombardo et al., 2006).

Mycorrhizal Colonization Assays

The ability of L. japonicus roots to be colonized by mycorrhizal fungi was tested using Glomus intraradices inocula from the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi. The plants were grown in pots containing inoculum mixed with sterile vermiculite (1:9, v/v). Daily watering with sterile water was supplemented weekly with sterile Hoagland solution No. 2 (Hoagland and Arnold, 1950), pH 6.8, containing the standard 6 mM KNO₃ and 4 mM Ca(NO₃)₂ but only 20 μM NH₄NO₃. The growth conditions were similar to the conditions used in the nodulation experiment. For the assay of colonization, the plants were gently removed from the soil at various time points and washed. The roots were cleared in 2.5% KOH at 90°C for 90 min, rinsed in distilled water, acidified in 1% HCl for 50 min, and stained in trypan blue at 90°C for 1 h. After digesting overnight in 50% glycerol, the roots were mounted on microscope slides in glycerol and observed with a microscope for fungal colonization. The roots were cut into approximately 1-cm segments and arranged in parallel on glass slides. The fungal colonization was analyzed by scanning across each segment once with a 2.1-μm field of view using a compound microscope. Over 100 segments were analyzed for each treatment.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Phylogenetic comparison of 70 plant apyrases.

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LNP in Symbiosis Signaling