

REVIEW



Perception of lipo-chitooligosaccharides by the bioenergy crop *Populus*

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ABSTRACT

Populus sp. is a developing feedstock for second-generation biofuel production. To ensure its success as a sustainable biofuel source, it is essential to capitalize on the ability of *Populus* sp. to associate with beneficial plant-associated microbes (e.g., mycorrhizal fungi) and engineer *Populus* sp. to associate with non-native symbionts (e.g., rhizobia). Here, we review recent research into the molecular mechanisms that control ectomycorrhizal associations in *Populus* sp. with particular emphasis on the discovery that ectomycorrhizal fungi produce lipo-chitooligosaccharides capable of activating the common symbiosis pathway. We also present new evidence that lipo-chitooligosaccharides produced by both ectomycorrhizal fungi and various species of rhizobia that do not associate with *Populus* sp. can induce nuclear calcium spiking in the roots of *Populus* sp. Thus, we argue *Populus* sp. already possesses the molecular machinery necessary for perceiving rhizobia, and the next step in engineering symbiosis with rhizobia should be focused on inducing bacterial accommodation and nodule organogenesis. The gene *NodU* *INception* is central to these processes, and several putative orthologs are present in *Populus* sp. Manipulating the promoters of these genes to match that of plants in the nitrogen-fixing clade may be sufficient to introduce nodulation in *Populus* sp.

ARTICLE HISTORY

Received 2 February 2021
Accepted 12 March 2021

Global climate change is an immediate threat to society and will pose an even more significant threat to future generations unless immediate action is taken to mitigate greenhouse gas emissions by transitioning to renewable energy sources.^{1,2} Biofuels provide the benefit of both reducing carbon dioxide emissions and sequestering atmospheric carbon into the soil.³ Critics argue that feedstocks for first-generation biofuels can lead to the displacement of crops for human consumption. Fortunately, second-generation biofuels (e.g., lignocellulosic materials from herbaceous, hardwood, and softwood crops) that do not displace crops are becoming equally viable biofuel options.⁴

Populus sp. is a leading woody biomass feedstock because it grows rapidly, can be cultivated on marginal lands, has a relatively high cellulose but low ash and extractive content, and is relatively easy to harvest, handle and store.⁵ The genomes of *Populus deltoides*, *P. euphratica*, *P. trichocarpa*, and the hybrid *P. tremula* × *alba* have been sequenced,^{6–8} and many *Populus* species can be readily transformed, including those with sequenced genomes.^{9,10} These breakthroughs enabled transgenic *Populus* lines that are less recalcitrant to plant cell wall deconstruction and saccharification but still perform well in field conditions.¹¹ Additionally, *Populus* sp. associates with beneficial soil microorganisms (e.g., mycorrhizal fungi), which increase its biomass production potential on marginal lands.¹² To capture the potential of microbial symbionts to enhance *Populus* sp. biomass production, we must expand our understanding of the molecular mechanisms that facilitate *Populus*

sp. interactions with beneficial microbes. Doing so could allow us to potentially engineer more efficient mycorrhizal associations and novel associations with nitrogen-fixing rhizobia for which *Populus* sp. is not a native host.

Mycorrhizal fungi are filamentous, soil-dwelling microorganisms that engage in mutualistic associations with the roots of nearly 90% of all terrestrial plant species.¹³ *Populus* sp. can engage in the two most ecologically and economically essential types: arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) associations.^{14,15} Due to the agricultural significance of AM associations, they have been studied more extensively than ECM associations. The known molecular mechanisms regulating AM associations have been thoroughly summarized in recent reviews.^{16–19} Although less is known about the molecular mechanisms regulating ECM associations, much has been discovered and reviewed previously.^{20–22} Here we only highlight the most recent advances in our understanding of the ECM association between *Populus* sp. and the model ECM fungus *Laccaria bicolor*, with particular emphasis on the role of lipo-chitooligosaccharides (LCOs) in the ECM establishment.

Many of the recent advances in our understanding of the *Populus*–*L. bicolor* symbiosis began with genomic studies. Analysis of 45 ECM fungal genomes (including *L. bicolor*) showed that ECM fungi possess fewer genes encoding plant cell wall-degrading enzymes than ancestral wood-decaying fungi.^{23,24} Using transcriptomics, Veneault-Fourrey *et al.* observed that different types of carbohydrate-active enzymes

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are expressed by *L. bicolor* during various colonization stages.²⁵ One of these, GH5-CBM1, has been shown to promote colonization.²⁶ These findings highlight how evolution has shaped the genome of ECM fungi such that they have primarily retained the carbohydrate-active enzymes necessary for manipulating the cell wall of their host plant to facilitate ECM development.

The analysis of the *L. bicolor* genome specifically led to the discovery and functional characterization of the small secreted peptide (SSP) MiSP7, which plays a crucial role in ECM development.^{27–30} Additional mycorrhiza-induced SSPs have since been identified, including MiSP7.6³¹ and MiSP8,³² involved in the early stages of mycorrhiza development in *Populus* sp. Intriguingly, *Populus* sp. also produces SSPs that are upregulated during its association with *L. bicolor*.³³ These studies on SSPs highlight a rich molecular dialog between the host plant and fungal symbiont that coordinates the early development of the mutualistic association.

Similarly, both *Populus* sp. and *L. bicolor* release small RNAs that probably target genes in their symbiotic partner.³⁴ The functional characterization of these small RNAs will likely provide exciting insights into the role of small RNAs in other plant-microbe interactions.³⁵ Additionally, phytohormones play a considerable role in plant-microbe interactions,³⁶ and the ECM symbiosis is no exception. Basso *et al.* (2020) recently evaluated the concentrations of phytohormones in ECM roots and the impact of their exogenous application on the development of the *Populus*–*L. bicolor* symbiosis.³⁷ They found that jasmonate, gibberellin, salicylate, and ethylene signaling play multifaceted roles in the establishment of this symbiosis.

Identifying small-molecule signaling pathways involved in ECM symbioses has remained elusive until recently. A G-type lectin receptor-like kinase *PtLecRLK1* was recently found to mediate *Populus* interaction with *L. bicolor*.³⁸ Surprisingly, when expressed in the non-ECM host *Arabidopsis*, *PtLecRLK1* confers the ability for a shallow Hartig net-like structure to form, providing the first evidence for the potential to engineer host

compatibility with ECM fungi in crop plants that cannot associate with mycorrhizal fungi. We found that, like AM fungi and rhizobia, the ECM fungus *L. bicolor* produces LCOs.³⁹ In compatible host plants, AM fungi and rhizobia use LCOs to activate the “common symbiosis pathway” (CSP).⁴⁰ Similarly, we found that the LCOs produced by *L. bicolor* activate the CSP in *Populus* and that components of the CSP are necessary for the full establishment of this ECM association.³⁹

It is important to note that some ECM hosts (e.g., *Pinus* and *Picea*) have lost critical components of the CSP.²⁰ Therefore, the CSP cannot be required for all ECM associations. However, we recently reported that LCOs are produced by *Pinus* and *Picea* fungal symbionts⁴¹ that may activate alternate non-CSP pathways in host plants or play roles beyond plant-fungal interactions. The presence of LCO-specific receptors in the model plant *Arabidopsis thaliana*,⁴² which like *Pinus* and *Picea* also lacks components of the CSP, indicates the existence of a CSP-independent signaling pathway for LCOs in plants. Beyond *Pinus* and *Picea* ECM fungal symbionts, we reported that nearly all filamentous fungi, including many diverse ECM fungi, produce LCOs, and that exogenous application of LCOs affects fungal development.⁴¹ Intriguingly, the structure of LCOs produced by the *Pinus* symbiont *H. cylindrosporum* is similar to those produced by *L. bicolor*.⁴¹ As such, we hypothesized that LCOs produced by *H. cylindrosporum* could activate the CSP in the non-host *Populus* spp.

To test this hypothesis, we followed the methods described in Cope *et al.*³⁹ to evaluate nuclear calcium spiking at the roots of *Populus tremula* × *alba* clone 717 in response to a suspension of *H. cylindrosporum* hyphal segments. We observed 105 nuclei across four root segments and found that 68 of them exhibited spikes in nuclear calcium concentration. Among these spiking nuclei, the mean number of spikes that occurred within 20 minutes of treatment with the hyphal suspension was 4.4 ± 0.4 (Figure 1a). For comparison, the *Populus* symbiont *L. bicolor* induced calcium spiking an average of 6.3 ± 0.4 times in 90 of 118 nuclei from four independent root segments (Figure 1b).

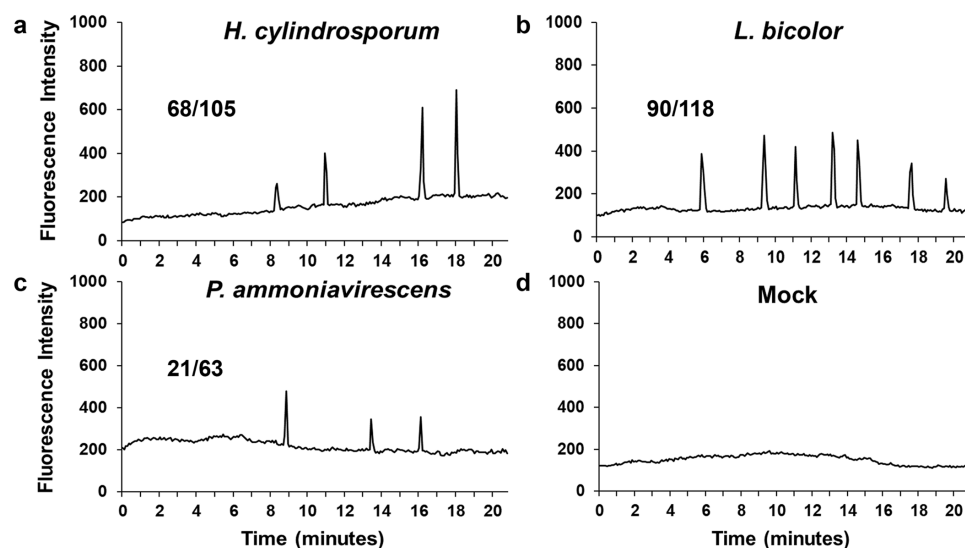


Figure 1. Summary of Ca^{2+} spiking in *Populus tremula* × *alba* clone INRA 717-1-B4 roots in response to ectomycorrhizal fungal hyphae. Representative plots of Ca^{2+} spiking in response to hyphae fragments from three species of ectomycorrhizal (ECM) fungi, *Hebeloma cylindrosporum*, *Paxillus ammoniavirescens*, and *Laccaria bicolor* or a mock treatment (water). The ratio of spiking nuclei to total nuclei observed is indicated for each treatment.

Also, we tested if *Paxillus ammoniavirescens*, another species of ECM fungi that colonizes *Populus* sp. and produces LCOs,⁴¹ could also induce calcium spiking. We observed that it did, although not as strongly as *L. bicolor* or *H. cylindrosporium* (21/63 spiking nuclei in three independent roots with an average of 3.9 ± 0.4 spikes in 20 min.; **Figure 1c**). No spiking was observed in nuclei from mock-treated roots (**Figure 1d**). These findings suggest that LCOs produced by ECM fungi that colonize *Populus* sp. and those that do not are both capable of activating the CSP. Accordingly, we propose that host-compatibility in ECM associations with *Populus* sp. is not determined by changes in LCO decorations.

Based on this conclusion, we must ask what regulates the difference in plant reactions to LCO producing symbionts and LCO producing non-symbiotic fungi or bacteria? LCO-induced calcium spiking in *Populus* sp. requires the same CSP components as legumes,³⁹ so it can be presumed that *Populus* sp. recognizes LCOs via the LYK/LYR receptor heterodimer conserved in most land plants.^{16,18} Discovering how *Populus* sp. discriminates between microorganisms may be the key to expanding its symbiotic potential.

In most host plants, rhizobia enter the root via trichoblasts (root hairs) and AM fungi via artrichoblasts. We would assume that AM fungi show this same preference in *Populus* sp.; however, we have demonstrated that both epidermal cell types respond to LCOs in *Populus* sp.³⁹ Therefore, the difference between *Populus* sp. and rhizobial host plants is not due to a lack of expression of the CSP in trichoblasts. The simplest explanation for different responses to symbiotic and non-symbiotic LCO producers would be differences in substitutions present in the LCO molecule that alter receptor-binding dynamics. This discrimination is observed in the response of legumes to rhizobia, where the presence of sulfate groups can cause the LCOs to trigger root hair curling in some species but not others.⁴¹ However, AM fungi appear to release a broad cocktail of LCOs with diverse substitutions, likely to enable a broad host range. Still, the conserved calcium spiking

response seems to be activated by sulfated or non-sulfated LCOs in all tested species, though some difference in binding specificity is observed.^{39,43,44} To ensure that rhizobia did not possess any undetected substitution that controlled the host range, we applied LCOs from two species of rhizobia with diverse substitutions (*Sinorhizobium meliloti* 2011 and *Rhizobium* sp. IRBG74)^{45,46} to *Populus tremula x alba* clone 717 roots, which produced a calcium spiking response indistinguishable from that of chemically synthesized LCOs or germinated spore exudates from AM fungi (**Figure 2**).³⁹

Roots treated with *Rhizobium* sp. IRBG74 or *S. meliloti* LCOs exhibited an average of 8.5 ± 0.6 and 11.8 ± 0.6 spikes per nuclei, respectively (**Figure 2a-figure 2b**). Those *Rhizobium* sp. IRBG74-derived LCOs continued to produce a strong response at 10^{-8} M (8.1 ± 0.6 spikes in 20 minutes; **Figure 3**), which would be expected as *Populus tremula x alba* 717 exhibits a preference for non-sulfated LCOs.³⁹ These responses are much lower than those seen in legumes, which produce calcium spikes in trichoblasts in response to as little as 10^{-14} M LCOs, likely due to changes in the receptor dissociation constant.⁴³

Several other hypotheses have been proposed to explain symbiont/non-host discrimination. First, all microorganisms release multiple microbe-associated molecular patterns (MAMPs), and integrating several pathways could tailor the response. Based on work in other species (including *Arabidopsis*, tobacco, tomato and rice) we would expect *Populus* sp. to recognize bacteria via peptidoglycan, lipopolysaccharides, exopolysaccharides, and flagellin.⁴⁷ It could differentiate between LCO producing bacteria and symbiotic fungi via a combinatorial perception of LCOs and other MAMPs. To test this, we applied cultures of both rhizobia species, pre-treated with host-derived inducers of LCO production, to *Populus tremula x alba* 717 roots. We observed a calcium spiking response in roots exposed to *Rhizobium* sp. IRBG74 (an average of 5.6 ± 0.2 spikes per nuclei within 20 minutes; **Figure 2c**), but a weaker response to *S. meliloti* (4.08 ± 0.2

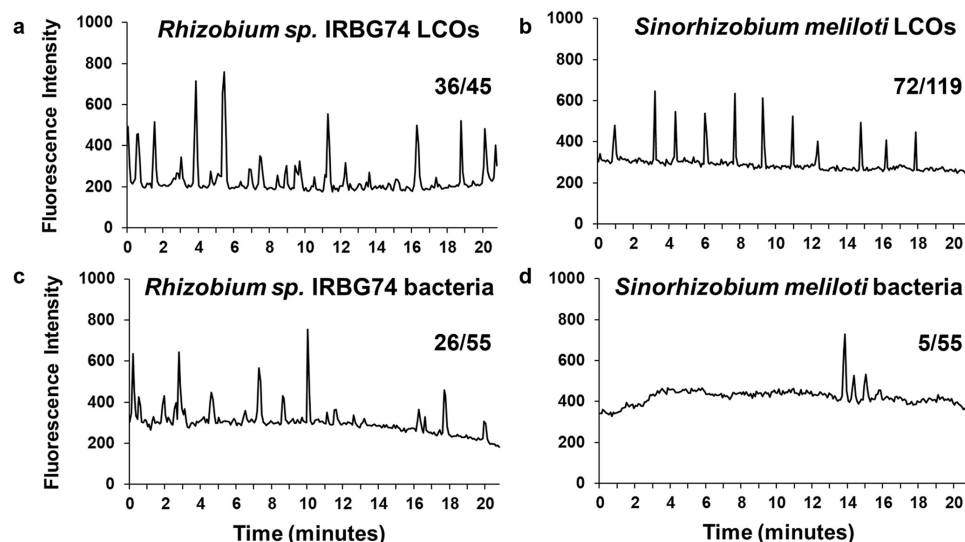


Figure 2. Summary of Ca^{2+} spiking in *Populus tremula x alba* clone INRA 717-1-B4 roots in response to rhizobia. Representative plots of Ca^{2+} spiking in response to 10^{-7} M LCOs from *Rhizobium* sp. IRBG74 and *Sinorhizobium meliloti* 2011 or the live bacteria (grown in VMM for 2 days with their activatory flavonoids, resuspended to an OD_{600} of 1.0 in an N/P/carbon depleted broth). The ratio of spiking nuclei to total nuclei observed is also indicated for each treatment.

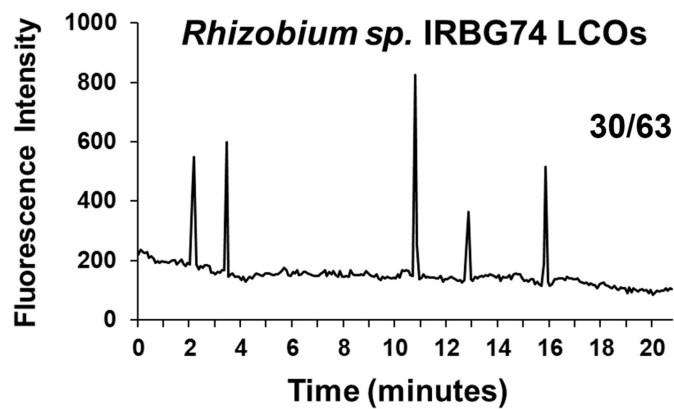


Figure 3. Summary of Ca^{2+} spiking in *Populus tremula* x *alba* clone INRA 717-1-B4 roots in response to *Rhizobium* sp. IRBG74 LCOs (10^{-8} M). Representative plot of Ca^{2+} spiking in response to 10^{-8} M LCOs from *Rhizobium* sp. IRBG74. The ratio of spiking nuclei to total nuclei observed is also shown.

spikes, though only 10% of nuclei showed at least 3 spikes; Figure 2d). Thus, we can conclude that rhizobial MAMPs do not inhibit the pre-symbiotic signaling via the common symbiosis pathway (CSP) in *Populus* sp. In *Rhizobium* sp. IRBG74, LCO production is triggered by the basal flavonoid naringenin, which poplar produces, so this initial signaling interaction could occur in nature. Further casting doubt on the multiple signals hypothesis, we have recently shown no apparent difference in LCO structures between many symbiotic and pathogenic fungi,⁴¹ the latter of which would be evolutionarily incentivized to minimize any difference in structural modifications.

Alternate explanations for symbiont discrimination include that the symbiont is more ‘forceful’ than often thought, suppressing host immunity via a battery of effectors and phytohormones,^{27,48–50} while an immune response suppresses symbiotic signaling for non-compatible symbionts. The broad host range of the Glomeromycotina is not what we would expect from a pathogenic fungus, but this could be due to the benefits of symbiosis selecting against the evolution of host resistance to the symbionts broad range effectors. There may also be symbiotic signals that we are as yet unaware of, such as the putative signals that trigger the D14L pathway in rice,⁵¹ that must combine with the output of calcium spiking to allow symbiosis. One possible ‘signal’ could be the penetration apparatus (hypopodium) size, which has been observed to be smaller in the Glomeromycotina than in pathogenic fungi and could thus lead to a different mechanical stimulation.^{52,53} However, it is unclear what would prevent a pathogen from evolving to replicate this if it was the only determinant.

The global nutrient status of the plant is known to regulate both fungal and bacterial symbioses; thus, in nutrient-replete conditions, LCO or other MAMP receptors are either inactive or trigger immune responses, and under nutrient-depleted conditions, they activate calcium spiking and the CSP. MAMP-triggered immunity is suppressed by a combination of CSP signaling (in response to LCO, chitoooligosaccharides and – at least in legumes – peptidoglycans) and symbiont effectors.^{27,43,54} This suppression of MAMP-triggered immunity is a risk (forcing the plant to rely on effector-triggered

immunity). Still, the nutritional benefits of symbiosis may be worth it when the plant has abundant carbon but low nutrient availability. Under this model, rhizobia produce the factors needed to induce the CSP in plants outside the nitrogen-fixing clade (NFC), but these plants lack coordination of the right gene set to allow proper infection or development of a root nodule. One apparent issue with this hypothesis are the examples of bacteria entering via crack entry (which could occur in any plant) and then forming infection structures directly into cortical cells using much of the same genetic machinery as Glomeromycotina fungi. As yet, there is no evidence that this occurs outside members of the NFC, suggesting there must be additional gating factors. Exopolysaccharides may play this role, as species-specific exopolysaccharides and putative exopolysaccharide receptors (LjEPR3/MtLYK10) are important for infection thread progression and bacterial survival inside the nodule.^{55,56} However, the *Medicago* and *Lotus* systems show notable differences, which may imply that these systems are a recent improvement to the RNS in these lineages, and more research is needed into the role of exopolysaccharides in basal RNS.

Understanding how *Populus* sp. distinguishes between LCO-producing microsymbionts and non-symbionts is crucial for engineering novel symbioses with nitrogen-fixing bacteria in this model system. Nitrogen-fixing symbiosis involves creating a nodule, a *de novo* root organ, which is the site for nitrogen fixation and has evolved within the NFC, which includes the orders Fabales, Fagales, Cucurbitales, and Rosales.^{57,58} Root nodule symbiosis (RNS) offers the most efficient mode of symbiotic nitrogen fixation.⁵⁹ With an ever-increasing demand for crop yield, nitrogen-fixing cereals are a “Holy Grail” for synthetic biologists.⁶⁰ One approach for achieving this goal is through engineering RNS in cereals.^{57,59,61} However, due to the phylogenetic distance of cereals from the NFC, the engineering has to be an iterative process involving intermediate species.⁵⁷ *Populus* sp. are members of the Malpighiales, much closer relatives to the NFC than monocotyledonous or Solanaceous crops (Angiosperm Phylogeny Website). Moreover, sequenced *Populus* sp. carry orthologs of the genes known to be required for the RNS.⁶² Combined with amenability to genetic manipulations, these traits make *Populus* sp. a promising proof-of-concept model for introducing the RNS to new hosts.

Studies focused on nodulation in legumes have shown that the calcium spiking induced by LCOs is decoded by a calcium and calmodulin-dependent kinase (CCaMK), which further activates a suite of transcription factors, including Nodule INception (NIN).^{58,63–66} NIN is a central regulator of bacterial accommodation and nodule organogenesis in both the rhizobia and *Frankia* nodule symbioses. Its absence is tightly correlated to loss of the symbiosis.⁶² Like *Populus* sp., Eudicot species outside the NFC have *NIN* orthologs, whereas monocots do not.⁶⁷ Recent studies have identified RNS-specific innovations in the *cis*-regulatory regions of *NIN* that arose within the NFC, suggesting promoter manipulation may be sufficient to introduce nodulation in *Populus* sp. without the need for the introduction of large transgene modules.^{67,68} Although *Populus* sp. does not produce nodules, various *Rhizobium* species have been found as endophytes in *Populus* via metagenomic or isolation approaches.^{69–71}

We have previously demonstrated that *Populus* responds to LCOs produced by its ECM and AM symbionts.³⁹ Here, we have shown that *Populus* has a similar response to LCO-producing fungi and bacteria with which no beneficial interactions have been observed. While we cannot rule out that such interactions are occurring unobserved in nature, this seems unlikely. This apparent inability to distinguish between LCO-producing organisms in the initial stages of the interaction, even if other MAMPs are available from live bacteria or fungi, challenges our current understanding of the CSP. This paradigm shift also presents opportunities, as the barrier to artificially expanding CSP-dependent symbioses to new host species may be less challenging than previously thought.

Funding

This work was supported by the National Science Foundation [2010789] and U.S. Department of Energy [DE-SC0018247].

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