A combination of chitooligosaccharide and lipochitooligosaccharide recognition promotes arbuscular mycorrhizal associations in *Medicago truncatula*

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Plants associate with beneficial arbuscular mycorrhizal fungi facilitating nutrient acquisition. Arbuscular mycorrhizal fungi produce chitooligosaccharides (COs) and lipochitooligosaccharides (LCOs), that promote symbiosis signalling with resultant oscillations in nuclear-associated calcium. The activation of symbiosis signalling must be balanced with activation of immunity signalling, which in fungal interactions is promoted by COs resulting from the chitinaceous fungal cell wall. Here we demonstrate that COs ranging from CO4-CO8 can induce symbiosis signalling in *Medicago truncatula*. CO perception is a function of the receptor-like kinases MtCERK1 and LYR4, that activate both immunity and symbiosis signalling. A combination of LCOs and COs act synergistically to enhance symbiosis signalling and suppress immunity signalling and receptors involved in both CO and LCO perception are necessary for mycorrhizal establishment. We conclude that LCOs, when present in a mix with COs, drive a symbiotic outcome and this mix of signals is essential for arbuscular mycorrhizal establishment.

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Plants are exposed to an array of microorganisms that include potential mutualists and pathogens and must monitor these interactions to coordinate appropriate responses, whether restriction of pathogens or promotion of mutualists. This decision-making is primarily regulated by two signaling pathways in the plant: symbiosis signaling that promotes microbial associations and immunity signaling that restricts them\(^1\). These pathways are predominantly associated with the regulation of intercellular and intracellular colonization of plant tissues, however, it is becoming clear that these pathways also impact on the microbiomes that form in association with plants\(^5,6\).

Primary recognition of fungi involves perception of chitinaceous molecules derived from the fungal cell wall. N-acetyl chitooctaose (CO8) is particularly active in promoting immunity signalling\(^2,7,8\), although other N-acetyl chitooligosaccharides (COs), including shorter chain COs such as N-acetyl chitotetraose (CO4), can activate immunity signalling, albeit at lower efficiencies\(^7,8\). Recognition of CO8 involves LysM-containing receptor-like kinases with the capability to bind directly to chitinaceous molecules through the LysM domains\(^9-16\). In both rice and Arabidopsis thaliana the perception of CO8 involves two LysM-containing receptors, one possessing an active intracellular kinase domain (CERK1\(^2,12,14\)) and one containing either an inactive kinase domain (LYK5 of A. thaliana\(^9\)) or a truncated intracellular domain (CEBIP in rice\(^11\)). A similar situation has been demonstrated to exist in legumes, with Medicago truncatula requiring the ACRERK1 homolog LK9 (renamed here MtCERK1) and the LLYK5 homolog LYY4 for CO8 activation of immunity signalling and appropriate resistance to fungal pathogens\(^7\). Perception of chitin by these receptors leads to the activation of plant defenses through production of reactive oxygen species (ROS), promotion of MAP kinases and activation of a calcium influx across the plasma membrane\(^2,7,9\).

Chitinaceous molecules also control beneficial fungal associations, with arbuscular mycorrhizal fungi producing both COs and lipochitooligosaccharides (LCOs)\(^17,18\), which possess an N-acetyl moiety on the terminal non-reducing sugar of the chitin chain\(^19\). Both LCOs and short chain COs (CO4 and CO5) can promote symbiosis signalling in M. truncatula with resultant oscillations in nuclear calcium levels\(^17,20\). Short chain COs activate symbiotic calcium oscillations in a range of species (M. truncatula, Lotus japonicus, carrot and rice\(^17,20\)) and in rice this is a function of the CO receptor CERK1\(^21\), that is necessary for appropriate arbuscular mycorrhizal colonization\(^22-25\). Arbuscular mycorrhizal fungi and rhizobial bacteria produce LCOs and the receptor complex in legumes involved in this perception contains two LysM-containing receptor-like kinases: LK3 and NFP in M. truncatula (NFR1 and NFR5 in L. japonicus)\(^26-33\). Mutations in these receptors abolish the interaction with nitrogen-fixing rhizobial bacteria, highlighting the essential role LCO perception plays in this bacterial symbiosis. In contrast, plants mutated in the LCO receptors have wild type or only marginally reduced colonization by arbuscular mycorrhizal fungi\(^23,34\).

Beneficial symbiotic microorganisms must evade plant defenses in order to colonize the root of their host plant. This may in part be regulated by effector molecules delivered into the cell from the symbiotic fungi or bacteria\(^35\). However, it has also been demonstrated that the plant actively suppresses immunity and this occurs following recognition of both CO4 and LCOs in A. thaliana and other plants\(^36\). In this work, we have used a combination of cell biology and genetics to characterize the relative contributions of COs and LCOs for establishment of arbuscular mycorrhizal associations in M. truncatula. We show that COs cannot be separated into those that activate symbiosis versus immunity responses, rather it appears that COs equally elicit symbiotic and immunity signalling, with longer chain COs, such as CO8, showing greater activity. Receptors responsible for both CO and LCO recognition are required in the establishment of arbuscular mycorrhizal associations and we show how the recognition of mixes of COs and LCOs emphasizes a symbiotic, over immunogenic, response.

**Results**

**CO8 activates symbiotic calcium signalling**. Symbiosis signalling involves the promotion of periodic calcium oscillations restricted to the nuclear region\(^37-39\). M. truncatula shows symbiotic calcium oscillations following treatment with either CO4 or LCOs\(^17,20\), but not to the immunity elicitor flg22 (0/18 epidermal cells showed calcium responses following treatment of 10\(^-5\) M flg22). We found that nuclear-associated calcium oscillations were activated following treatments with CO8 (Fig. 1a, b), previously thought to function primarily as an immunity signal\(^40\).

To test the degree to which other CO molecules activate symbiotic signalling we assessed the induction of nuclear calcium oscillations by all CO molecules between CO2 and CO8. CO4, CO5, CO6, CO7 and CO8 all activate nuclear calcium oscillations, with comparable activities when applied at 10\(^-8\) M. However, neither CO2 nor CO3 could activate nuclear calcium oscillations (Supplementary Table 1).

The CO8-induced nuclear calcium responses of roots in whole plants of M. truncatula show a periodicity similar in nature to those induced by CO4 (Fig. 1a). Dose response curves that assess the number of cells responding with nuclear calcium oscillations across a range of elicitor concentrations, indicate that CO8 is more active in M. truncatula roots than CO4 (Fig. 1c). CO4 can induce immunity signalling in M. truncatula\(^1\), but the degree of the CO4 response is much less than CO8 and this is comparable to the reduced activity of CO4 in promotion of symbiosis signalling. In contrast, SmlCOS are much more active than either CO4 or CO5 for induction of nuclear calcium oscillations (Fig. 1c), but show no induction of ROS or MAPks, markers of immunity signalling\(^7\). The LCO produced by the mycorrhizal fungus *Rhizophagus irregularis* (NS-LCO) shows an activity within a similar range as CO4/C08, but is slightly less active than either molecule (Fig. 1c). The concentrations of CO8 required for the induction of symbiosis signalling are comparable to those required for induction of immunity signalling\(^7\), implying that the receptors involved in CO8 perception for immunity or symbiosis signalling must have comparable activation kinetics.

It was previously assumed that CO8 only functions as an immunity elicitor and therefore, it was very surprising to see CO8 induction of symbiosis signalling. To validate that this response was indeed a function of CO8 we first tested the purity of our CO8 samples and found that they were not contaminated with either CO4 or CO5 (Supplementary Fig. 1a). Plant roots exude a number of chitinases and it is possible that treating roots with CO8 leads to an accumulation of shorter chain COs as degradation products of CO8 and the resultant short chain COs could then activate symbiosis signalling. CO8 treated on *M. truncatula* roots does indeed get broken down quickly, with a 50% reduction in total CO8 levels after 10 min incubation on *M. truncatula* roots (Supplementary Fig. 1b). The chitinase inhibitor acetazolamide\(^41\) reduces the rate and degree of this CO8 degradation (Supplementary Fig. 1b) and consistent with this we found that co-treatment of acetazolamide with CO8 enhanced the activation of immunity signalling as measured by the activation of ROS (Supplementary Fig. 2a). The symbiotic response to CO8 was also enhanced by co-treatment with acetazolamide: we found a significantly reduced activation time for CO8 promotion of nuclear calcium oscillations.
instead took an indirect approach to assess for the presence of CO8 in R. irregularis exudates. We treated Arabidopsis leaf discs with R. irregularis germinated spore exudates and observed a rapid ROS burst indicative of the activation of immunity signalling. This ROS response was greatly reduced in Aterck1 mutants revealing that the chitinaceous elicitor of AterCK1 must be present in these exudates of R. irregularis (Supplementary Fig. 3a). We conclude that chitinaceous elicitors are produced by arbuscular mycorrhizal fungi and they promote both symbiosis and immunity signalling.

\textbf{MtCERK1 and LYR4 are receptors for CO8.} To validate that these calcium responses were a function of symbiosis signalling we assessed CO8-induced calcium responses in plants mutated in components of the symbiosis signalling pathway necessary for the establishment of nuclear calcium oscillations: DMI1 and DMI2\textsuperscript{42–44}. Both dmi1 and dmi2 mutants abolished CO8-induced nuclear calcium oscillations (Fig. 2a), indicating a function for symbiosis signalling in this CO8-induced calcium response. DMI2 encodes a receptor-like kinase able to associate with LCO receptors in L. japonicus\textsuperscript{45,46}. Interestingly, while this receptor is essential for both CO and LCO induction of symbiotic calcium oscillations, it is not required for CO8-induced defence responses (Supplementary Fig. 4). Mutations in dmi1 and dmi2 abolish symbiotic signalling and beneficial microbial associations\textsuperscript{17,20,42–44} but have no effect on immunity signalling and little or no effect on pathogen interactions\textsuperscript{47–50}. We therefore conclude that CO8-induced nuclear calcium oscillations are a function of symbiosis signalling.

The LCO receptors NFP and LYK3 were not required for CO8 induction of calcium oscillations (Fig. 2a and Supplementary Fig. 5a), implying the involvement of an alternative receptor complex. \textit{M. truncatula} LYR4 and MtCERK1 act as CO8 receptors for activation of immunity signalling and are necessary for restriction of the fungal pathogen Botrytis cinerea\textsuperscript{7}, as well as immunity responses to \textit{R. irregularis} germinated spore exudates (Supplementary Fig. 3b). To assess if these CO8 receptors were also necessary for the activation of symbiotic responses we crossed these receptor mutants into the calcium reporter Yellow cameleon YC3.6 to measure the activation of symbiotic calcium responses. We found that MtCERK1 was essential for CO8-induced symbiotic calcium oscillations in both trichoblasts (epidermal root hair cells) and atrichoblasts (epidermal root cells lacking root hairs; Fig. 2a and Supplementary Fig. 5a). Surprisingly, LYR4 was not required for CO8-induced calcium responses in atrichoblasts, but was required for CO8-induced calcium oscillations in trichoblasts (Fig. 2a and Supplementary Fig. 5a), suggesting additional receptors in atrichoblasts that may compensate for the absence of LYR4. Complementation of Mterck1 and lyr4 with their respective wild-type genes reinstated CO8 activation of symbiotic calcium oscillations (Supplementary Table 3). We have previously demonstrated that MtCERK1 and LYR4 are required for activation of immunity signalling in \textit{M. truncatula} by both CO4 and CO8\textsuperscript{7}. In a similar fashion, we found that MtCERK1 was essential for CO4-induced calcium oscillations in both trichoblasts and atrichoblasts, while LYR4 was required for CO4-induced calcium oscillations in trichoblasts, but not in atrichoblasts (Supplementary Fig. 5b, c). We conclude that MtCERK1 and LYR4 are responsible for CO8 and CO4 activation of both immunity and symbiosis signalling.

Our genetic evidence implies a function for MtCERK1 and LYR4 in the perception of both CO4 and CO8. To validate these observations we generated beads fused to either CO4 or CO8, using biotinylated versions of CO4 and CO8, attached to Streptavidin beads\textsuperscript{51}. MtCERK1 showed strong binding to CO8 and instead took an indirect approach to assess for the presence of CO8 in R. irregularis exudates. We treated Arabidopsis leaf discs with R. irregularis germinated spore exudates and observed a rapid ROS burst indicative of the activation of immunity signalling. This ROS response was greatly reduced in Aterck1 mutants revealing that the chitinaceous elicitor of AterCK1 must be present in these exudates of R. irregularis (Supplementary Fig. 3a). We conclude that chitinaceous elicitors are produced by arbuscular mycorrhizal fungi and they promote both symbiosis and immunity signalling.
and weaker binding to CO4, while LYR4 showed some binding to CO8, but no binding to CO4 (Fig. 3a). The MtCERK1 binding to CO4 beads could be eluted with addition of either CO4 or CO8, but not SmLCO (Fig. 3b). However, CO8 was more effective than CO4 at eluting MtCERK1 from the CO4 beads. The LYR4 binding to CO8 could only be competed with addition of CO8 (Fig. 3b). From this work, we conclude that MtCERK1 can bind both CO4 and CO8, but has a preference for CO8, while LYR4 can bind CO8, but it appears with less efficiency than MtCERK1.

**Fig. 2** CO8 and PGN activate calcium oscillations dependent on DMI1, DMI2, MtCERK1 and LYR4. Representative calcium traces of *M. truncatula* trichoblasts of lateral roots responding to 10⁻¹⁸ M CO8 (**a**) and 0.8 mg/ml PGN (**b**) in wild type and different mutants. The traces denote the ratio of YFP to CFP in arbitrary units. Numbers indicate cells responding compared to total cells analyzed.

**Fig. 3** LYR4 and MtCERK1 bind to COs and PGN. **a** LYR4 and MtCERK1 binding with streptavidin beads fused to either CO4 or CO8. CK: streptavidin beads alone. **b** MtCERK1 binding with CO4 beads can be eluted by addition of CO4 and CO8. LYR4 binding with CO8 beads can be eluted with CO8, but not CO4. **c** LYR4 and MtCERK1, but not DMI2, bind to insoluble PGN and **d** bound proteins can be eluted by CO8 and the soluble fraction of PGN, but not SmLCO. The protein expression level of LYR4 and MtCERK1 used in the binding assay is shown as input.

**CO8 activates symbiotically relevant gene expression.** To further explore the relative overlap between immunity and symbiosis signalling, we explored the transcriptional profiles of roots treated with either SmLCO, CO4, CO8 or flg22 (Supplementary Data 1). *R. irregularis* produces a sulphated LCO very similar to the predominant LCOs produced by *S. meliloti*, that also shows similar elicitation activity as SmLCO for induction of symbiotic calcium oscillations in *M. truncatula*. In this study we used SmLCO as a measure of the LCO-induced response. Of the genes that were significantly upregulated by greater than two-fold we observed a large overlap between CO8 and flg22 (54% of CO8-induced genes were also induced by flg22; Supplementary Fig. 6a), with much less overlap between SmLCO and flg22 (25% of SmLCO-induced genes were upregulated in flg22 treatments; Supplementary Fig. 6a). By contrast, 51% of genes induced by SmLCO were also activated by CO8 (Supplementary Fig. 6a), revealing a sizable overlap between the CO8 and SmLCO responses.

Flg22-induced genes show induction by COs, less induction by SmLCO and very little induction by mycorrhizal fungi (Fig. 4a). We conclude that these flg22-induced genes most likely represent the immunity response and this is supported by the presence of a number of genes known to function in plant defenses among this group (Supplementary Fig. 7). A second group of genes are induced by CO8, SmLCO and mycorrhizal colonization (Fig. 4a), but not flg22. These include a number that are known to function during mycorrhizal colonization, for instance Vapyrin, HA1, D27, PUB1 and a symbiotic subtilase (Supplementary Fig. 7). To assess the dependency of these gene expression changes on the CO and LCO receptors, we analyzed responses of CO4, CO8 and SmLCO in *Mtcerk1, lyr4* and *nfp* mutants. For this work, we generated a new *nfp* mutant allele in the *M. truncatula* ecotype R108 (Supplementary Fig. 8). This *nfp* allele allowed ease of comparison with *Mtcerk1* and *lyr4*, that are also in the R108 ecotype. 86.43% of the genes that were activated by CO8 were significantly affected by *nfp*. A second group of genes is only activated by CO8 and *nfp* (Supplementary Fig. 7), which we interpret as a direct response to the LCOs and missing the intermediate steps in the CO8 signal transduction pathway.

**Fig. 4** Gene expression changes in wild type and different mutants. **a** Numbers indicate cells responding compared to total cells analyzed. **b** Gene expression changes in wild type and different mutants. **c** Gene expression changes in wild type and different mutants. **d** Gene expression changes in wild type and different mutants. **e** Gene expression changes in wild type and different mutants. **f** Gene expression changes in wild type and different mutants. **g** Gene expression changes in wild type and different mutants. **h** Gene expression changes in wild type and different mutants. **i** Gene expression changes in wild type and different mutants. **j** Gene expression changes in wild type and different mutants. **k** Gene expression changes in wild type and different mutants. **l** Gene expression changes in wild type and different mutants. **m** Gene expression changes in wild type and different mutants. **n** Gene expression changes in wild type and different mutants. **o** Gene expression changes in wild type and different mutants. **p** Gene expression changes in wild type and different mutants. **q** Gene expression changes in wild type and different mutants. **r** Gene expression changes in wild type and different mutants. **s** Gene expression changes in wild type and different mutants. **t** Gene expression changes in wild type and different mutants. **u** Gene expression changes in wild type and different mutants. **v** Gene expression changes in wild type and different mutants. **w** Gene expression changes in wild type and different mutants. **x** Gene expression changes in wild type and different mutants. **y** Gene expression changes in wild type and different mutants. **z** Gene expression changes in wild type and different mutants.
dependent on MtCERK1, while 65.62% of these genes showed LYR4 dependence (Fig. 4a). The reduced dependency of the CO8-response on LYR4, as oppose to MtCERK1, is consistent with the observation that CO8-induced calcium oscillations are maintained in atrichoblasts (Supplementary Fig. 5a). Surprisingly, there was also a function for NFP in the CO8 response, with 47.95% of the CO8-induced genes being NFP-dependent. The degree of transcriptional response to CO4 was greatly attenuated compared to CO8, consistent with CO8 being a more active elicitor. However, there are some genes induced specifically by CO4, implying a degree of specificity in the CO4 response (Fig. 4a). The CO4 transcriptome was highly dependent on both MtCERK1 (80.16% of genes) and NFP (86.96% of genes), with less dependency on LYR4 (72.27% of genes). The majority (93.42%) of genes induced by SmLCO were dependent on NFP. MtCERK1 and LYR4 also contribute to the SmLCO response but by a lower degree, 46.03% and 47.17% respectively. While these analyses, along with previous work, support a primary function for MtCERK1 and LYR4 in the CO response and NFP in the LCO response, they also reveal contributions from all receptors in CO and LCO perception.

We validated these gene inductions using quantitative reverse transcription polymerase chain reaction (qRT-PCR) of Vapyrin, MtLYK10, HA1 and D27 induction. Included in this analysis were the Mtcerk1, lyr4 and nfp receptor mutants, but also mutants in the symbiosis signalling components dmi1 and dmi2. We observed CO8 and SmLCO induction of Vapyrin, MtLYK10, HA1 and D27 using qRT-PCR, and in all cases this induction was...
dependent on DMI1 and DMI2 (Fig. 4b, c and Supplementary Fig. 6b, c), revealing a function for symbiosis signalling in these gene expression changes. All genes induced by CO8 were MtCERK1 and LYR4 dependent and in a few cases NFP dependent, while SmLCO induction of these genes was NFP dependent, but MtCERK1/LYR4 independent (Fig. 4b, c and Supplementary Fig. 6b, c). Interestingly, the defense-related genes induced by CO8 were dependent on MtCERK1 and LYR4, but independent of DMI1 and DMI2 (Fig. 4d, e and Supplementary Fig. 6d, e). DMI2 is not required for plant immunity (Supplementary Fig. 4), suggesting that the differential activation of immunity or symbiosis signalling by MtCERK1/LYR4 might be explained by the presence of DMI2 as a co-receptor. Our work reveals that CO8 activates genes associated with both immunity and symbiosis and this correlates with its ability to activate both immunity and symbiosis signalling.

Recognition of COs with LCOs promotes symbiosis over immunity. *R. irregularis* appears to produce both COs and LCOs and we would expect that a combination of these signals will be presented to root cells as the fungus approaches. It has already been demonstrated that LCOs can inhibit the degree of immunity signalling induced by CO8 or Olig2236. Here we further explored the effect of these signals when presented in combination. Consistent with previous work26, when SmLCO was combined with CO8 we found a reduction in the resultant ROS and MAPK responses (Fig. 5a, b), within 15 min of treatment with SmLCO (Fig. 5a), qRT-PCR analysis of two genes induced by Olig22 and CO8 (Fig. 5c), a PR10 and a chitinase, revealed that co-treatment with SmLCO inhibited CO8 induction of these genes (Fig. 5c), in disagreement with a recent report56. The combination of LCOs and COs inhibits defense-related gene expression, but these two signals act synergistically to enhance the degree of symbiotic gene expression, as measured using qRT-PCR of Vapyrin and HA1 (Fig. 5c).

To demonstrate whether LCO suppression of plant immunity is a function of the symbiosis signalling pathway, we assessed SmLCO modification of plant immunity in mutants defective in symbiosis signalling. Only NFP, but not other genes in this pathway, were necessary for SmLCO suppression of immunity (Fig. 6a), suggesting an independent signalling function downstream of NFP, that is different to the signalling pathways. Overexpression of NFP alone cannot inhibit MtCERK1-induced cell death (Supplementary Fig. 9) and treatment with the protein synthesis inhibitor cycloheximide blocked the SmLCO suppression of immunity signalling (Supplementary Fig. 10a), pointing at novel protein synthesis being involved. LCO treatment enhances root susceptibility to the pathogenic oomycete *Phytophthora palmivora*, and this was dependent on NFP, but not other components of symbiosis signalling (Fig. 6b, c). Consistent with a previous report49,57, nfp showed increased susceptibility to *P. palmivora* (Fig. 6b, c) and in addition we found that mutants of Mtcerk1 and lyr4 showed greater susceptibility (Supplementary Fig. 11), implying roles for these receptors in pathogenic root associations.

Our work indicates that when presented together LCOs alter the nature of the CO response towards a symbiotic outcome, with reduced immunity and enhanced symbiosis signalling. This suggests that such a combination of signal perception will be important during the interaction with mycorrhizal fungi. NFP is necessary for all LCO responses23,24, whether of fungal or bacterial origin, implying that this receptor may have functions beyond simply a role in rhizobial interactions, as already demonstrated26,27. To test the importance of the mix of COs and LCOs we generated a number of double mutant combinations that combined mutations in the LCO receptors NFP and LYK3 and the CO receptors MtCERK1 and LYR4. Because LYK3 and LYR4 are in close proximity on the *M. truncatula* genome it was not possible to generate the lyk3/lyr4 double mutant. All lines that carried a mutation in *MtCERK1* showed significant reductions in mycorrhizal fungal colonization at both three and five weeks post inoculation with *R. irregularis* spores, in support of recent work24. In contrast, mutation of *LYR4* alone appeared to have no effect (Fig. 7a). This differential dependence on the CO8 receptors likely reflects the cell specific nature of *lyr4*, since mycorrhizal fungi preferentially colonize the root hairs of the *lyr4* mutant. We found that the dual suppression of immunity and symbiosis by double mutant combinations is dependent on the CO8 receptors likely reflecting the cell specific nature of *lyr4*, since mycorrhizal fungi preferentially colonize the root hairs of the *lyr4* mutant.

![Image](https://example.com/image.png)

Fig. 5 A combination of CO8 and SmLCO can promote symbiosis and inhibit immunity signalling. **a**. *M. truncatula* wild-type roots were pretreated with 10−8 M SmLCO for different time points (minutes) before incubation with 10−6 M CO8 for induction of ROS. The significant groupings were calculated with a Mann–Whitney Rank Sum Test (mean ± s.e.m., n = 6, P < 0.05). This experiment was repeated twice with similar results. **b**. MAPK activation in wild type and nfp roots induced by 10−8 M CO8 with or without 15 min pretreatment of 10−8 M SmLCO. **c**. Equivalent treatment as b for qRT-PCR detection of gene expression testing immunity reporters *PR10* and a Chitinase and symbiosis reporters *HA1* and *Vapyrin* in response to CO8 and PGN, with or without SmLCO. Relative fold change compared to individual water treatments are shown. Letters denote statistically significant groupings calculated with Mann–Whitney Rank Sum Test (mean ± s.e.m., n = 3; P < 0.05), this experiment was repeated twice with similar results.
Peptidoglycan is also a dual immunity/symbiotic elicitor. Like chitin, PGN that is present in bacterial envelopes, possesses N-acetyl glucosamine residues, but additional sugars are present that are linked through a combination of bonds to create a much more complex network of sugar moieties than chitin. PGN perception in Arabidopsis and rice involves CERK1 and LYR4 and therefore we explored whether PGN may mirror CO8 in acting as a dual immunity and symbiosis elicitor. PGN activates nuclear calcium oscillations in a manner comparable to CO8, as well as the receptors MtCERK1 and LYR4 (Fig. 2b and Supplementary Fig. 5d). Like CO8, we observed some calcium oscillations in atrichoblasts of lyr4, as well as Mtcerk1, but in the case of PGN, there still appeared to be a reduction in the response of atrichoblasts in these mutants (Supplementary Fig. 5d, e). We found that both MtCERK1 and LYR4 could bind to PGN (Fig. 3c) and this binding could be eluted with addition of either CO8 or PGN (Fig. 3d). Like CO8, PGN acts as an immunity elicitor in M. truncatula in a manner dependent on MtCERK1 and LYR4 (Supplementary Fig. 12) and this induction of immunity signalling could be suppressed by addition of LCOs (Supplementary Fig. 10b, c). We conclude that PGN appears to function in a similar fashion as CO8, acting as both an immunity and a symbiotic elicitor, however, unlike mycorrhization, we were unable to observe any phenotypes during rhizobial colonization in the Mtcerk1 or lyr4 mutants.

Discussion

Symbiosis signalling is preserved across diverse plant species and is necessary for perception of arbuscular mycorrhizal fungi and their accommodation in plant roots. This signalling pathway has also been recruited during the evolution of nitrogen-fixing symbioses in legumes and actinorhizal species, that use this pathway to perceive LCOs produced by rhizobial bacteria or an unknown signalling molecule produced by Frankia bacteria. The presence of the genetic components that make up symbiosis signalling correlate very closely with the capability to enter symbiotic associations: plant species that have lost arbuscular mycorrhizal associations, lose the symbiosis signalling pathway, suggesting the signalling pathway is redundant in the absence of beneficial microbial associations. The role of symbiosis signalling during symbiotic associations is absolute: mutations in this pathway completely abolish colonisation of arbuscular mycorrhizal fungi and rhizobial bacteria. No such major role has yet been found for this pathway during pathogen or parasitic associations, instead symbiosis genes or their homologs appear to play only marginal roles in colonisation by a very limited set of pathogens or parasites. We therefore conclude that symbiosis signalling functions primarily in the establishment of mutualistic associations and despite the presence of this signalling pathway over hundreds of millions of years of co-evolution, there is very little evidence that pathogens or parasites have utilised this signalling pathway to promote their own colonisation.

The receptor NFP is essential for all LCO responses in M. truncatula and required for associations with rhizobial bacteria. However, the effect of NFP is broader, since it contributes to resistance against both oomycete and fungal pathogens. Here we demonstrate that NFP also contributes to colonization by arbuscular mycorrhizal fungi, but this effect can only be observed when combined with the Mtcerk1 mutant. In addition to the role of NFP in LCO responses, we have observed some dependence on NFP in the CO response. This is particularly apparent in the CO4 transcriptional response and the CO4 induction of calcium oscillations, but we also observed some dependence on NFP in the CO8 transcriptional response. Hence, NFP may be involved in...
the recognition of mycorrhizal-produced LCOs\textsuperscript{18}, a role analogous to its function during rhizobial associations, or alternatively, NFP may act in concert with other receptors during recognition of COs.

Arbuscular mycorrhizal fungi appear to be able to promote symbiosis signalling in \textit{M. truncatula} through a combination of COs and LCOs. The additional presence of COs in mycorrhizal fungal exudates allows activation of the symbiosis signalling pathway even when perception of LCOs is restricted and based on the fact that \textit{nfpm} mutants show no LCO responses, but maintain wild-type levels of mycorrhizal infection, suggests that CO activation of the symbiosis signalling pathway is sufficient to sustain this symbiosis. Arbuscular mycorrhizal fungi show enhanced production of at least short chain COs in response to the plant signal strigolactone\textsuperscript{17}, suggesting that COs may be actively produced by these fungi as signals to the plant. \textit{M. truncatula} has many LysM-RLKs in addition to those studied here and at least some of these additional receptors bind LCOs\textsuperscript{67}. Hence, multiple receptors may contribute to mycorrhizal recognition of COs, LCOs and possibly other signalling molecules and this may explain the remaining fungal colonisation of the \textit{nfp/Mtcerk1} double mutant. It is possible that a similar situation exists during colonisation by rhizobial bacteria, with both PGN and LCOs able to activate symbiosis signalling.

It has previously been thought that differential chitinaceous molecules show different activities: short chain COs, such as CO4 and CO5 were demonstrated to activate symbiosis signalling\textsuperscript{17,20}, while long chain COs, such as CO8, induced immunity signalling\textsuperscript{21,22}. Such a model implies different perception of COs of different chain lengths and the discrimination of symbiotic and pathogenic fungi through such differential recognition. Here we show that this separation of CO molecules does not exist: all COs from CO4 to CO8 activate both symbiosis and immunity signalling, at least in \textit{M. truncatula}. CO8 shows the greatest level of elicitation\textsuperscript{7,13} and consistently the CO receptors show preferential binding to CO8 over CO4. Promotion of symbiosis signalling\textsuperscript{17,20} and immunity signalling\textsuperscript{7,8} and the suppression of immunity signalling\textsuperscript{36} by CO4 appears contradictory, but it may be that the nature of the response is conditional on the status of the plant at the time of elicitation.

Plant perception of COs and PGN act as a surveillance mechanism for the recognition of fungi and bacteria within the vicinity of the root, but the presence of these elicitors does not differentiate symbiotic from pathogenic microorganisms. Consistently, the plant also does not appear to discriminate between symbiosis versus immunity responses based on CO or PGN perception alone. Our work has focused on \textit{M. truncatula}, but parallel research points at similar situations in other species: silencing of LysM RLKs in \textit{Parasponia} and tomato is detrimental to mycorrhizal colonisation\textsuperscript{58,69}, \textit{cerk1} mutants of rice show delayed colonisation of mycorrhizal fungi and reduced responses to CO4\textsuperscript{21,22} and \textit{CERK1} of banana has roles in both symbiosis and immunity\textsuperscript{25}. LCOs appear to play a pivotal role in symbiosis, with the capability to suppress immunity signalling\textsuperscript{36,70} and enhance symbiosis signalling. We suggest that a combination of COs and LCOs promotes a symbiotic outcome, while perception of COs alone preference immunity (Fig. 7d). Critical in this decision-making process is the receptor-kinase DM12, that promotes symbiosis signalling in response to both COs and LCOs, but appears to play no role in the activation of immunity signalling. The decision to enter a symbiotic interaction may result from the activation of not only symbiosis signalling, but also
other parallel signalling processes and the demonstration that the karrakin receptor is required for nycorrhiza colonisation in rice highlights that additional signalling functions may occur in the plant. Our work reveals that plants choose to encourage or restrict fungal colonisation, not based on the recognition of single signalling molecules, but rather through an integration of the mix of signalling molecules perceived.

**Methods**

**Plant materials and growth conditions.** *M. truncatula* cv. Jenalong A17 and R108 were used as wild type, mutants *nfp* (*nfp*-1), *lyk3* (*lyk3*-1), *dm1-2* (*dm1*-2, *dm1*-3) and *dm3-1* were reported previously.

The Tnt1 transposon insertion mutants *lyr4-1* (NF10265), *lyr4-2* (NF15280), *nfp* (NF7796) and *Mtcerk1* (NF16753) were obtained from the Samuel Roberts Noble Foundation collection.

Yellow Cameleon (YC) 3.6 was incorporated into these mutants by crossing with an existing stable *M. truncatula* YC3.6 line.

**M. truncatula** seeds were treated with concentrated sulfuric acid (Thermo Fisher Scientific) for 10 min, followed with four times wash in sterilized water. The seeds were then surfaced sterilized in 10% sodium hypochlorite for 2 min and rinsed in sterilized water five times. The sterilized seeds were transferred to 1.5% water agar plates and kept in the cold for 2 days and then grown on room temperature overnight before germination. Seedlings were then either grown on water agar plates and kept in the cold for 2 days and then grown on room temperature or cultivated in a 1:1 mixture of sterilized aluminium silicate and sharp sand (Oil-dri Ltd and Berrycroft Horticulture). Plants were incubated in controlled environment rooms at 22 °C and 80% humidity (16 h photoperiod and 8 h dark) without humidity and light for 1 week before germination. The seedlings were then transferred to a growth room at 21 °C with a 10 h photoperiod. Five-week old *M. truncatula* plants were grown in a glasshouse for protein expression.

**Nuclear calcium imaging.** For calcium analyses *M. truncatula* seedlings were grown on Buffered Nodulation Media (BNM) agar with 100 mM AVG (*Sigma- Aldrich*) until lateral roots emerged. All calcium analyses were performed on lateral roots since these have been demonstrated to show greater sensitivity to symbiotic signals than the primary root. For the chitinase inhibition assay, *M. truncatula* lateral roots were treated with 0.1% DMSO or 1 mM Acetazolamide (*Sigma- Aldrich*; dissolved in DMSO) for 30 min before challenging with different concentrations of CO8 to test nuclear calcium oscillations. The lateral roots were fixed in a small chamber on a cover glass using vacuum grease, with 500 μl of BNM buffer. The roots were then treated with COs, LCOs, or 8 M CO8. The lateral roots were fixed in 45% formamide for 2 h and washed with 1x PBS buffer, then stained in 0.5 μM Alexa Fluor 488 dye (Molecular Probes) for 6 h. Images were taken using a Leica DM6000 light microscope or for higher resolution images a Zeiss LSM 780 confocal microscope.

**Gene expression analysis.** *M. truncatula* wild type and mutants were grown on BNM plates with 100 mM AVG for 4 days, the plants were then transferred to liquid BNM with control DMSO or elicitors (10−7 M fog22, 10−7 M CO4, 10−7 M CO8 or 10−8 M SmLCO) for 6 h. We used eight plants for each treatment and three biological replicates for each time point. Plants were harvested and frozen in liquid nitrogen. Total RNA was extracted from root tissues using the Qiagen RNeasy Plant mini kit, DNA was removed by treatment of RNA with RNase-free DNase according to the manufacturer's instructions. An aliquot of each total RNA sample was used to determine RNA concentration and purity assessed on Nanodrop 2000 (Thermo Fisher Scientific, USA). RNA integrity was analyzed on the 2100 Bioanalyzer using RNA 6000 Nano LabChip Kits (Agilent Technologies).

Library preparation was performed as described for the TruSeq Stranded mRNA HT kit and sequencing of the library was run on a NextSeq500 sequencing system (Illumina). The resulting reads were processed, quality checked, and mapped to the *M. truncatula* (Mt4v0.4) genome sequence and analyzed for differential gene expression in different comparisons using the edgeR statistical package. Fold changes ≥ 2 and False Discovery Rate (FDR) corrected p-values ≤ 0.05 were used as thresholds for the gene to be identified as being differentially expressed (Supplementary Data 1). The raw data from the RNA sequencing has been deposited at the NCBI Sequence Read Archive (SRA) with accession number SRP097705.

**Plasmid construction and complementation.** All the constructs used in this research were created using a golden gate cloning strategy. For *lyr4* and *Mtcerk1* complementation, the constructs containing synthesized coding sequences of LYR4 and *Mtcerk1* with 35S terminators under the control of Lyr4 and *Mtcerk1* promoters were transformed into *lyr4* and *Mtcerk1* mutants expressing YC3.6 by hairy root transformation. Plant roots with strong DsRed fluorescence were selected to measure calcium oscillations after treatment of 10−8 M CO8. For *nfp* (NF7796), complementation, a full-length genomic clone of *Homologous transformation* was used as a symbiosis marker genes while pathogenesis-related proteins or PR proteins (*Medtr1g22904), *PR4* (Medtr1g080800), a plant chitinase (*Medtr2g099470*) and a plant glutathione S-transferase GST (*Medtr3g467420*) were considered as defense marker genes (all were induced by *fog22*). Expression data were analyzed from the average of threshold cycle (Ct) value, using an *M. truncatula* endogenous histone 2A gene as a reference and fold induction calculated for treatment of elicitors relative to treatment with DMSO. The qRT-PCR primers used can be found in Supplementary Table 4.

**Reactive oxygen species.** Leaf discs (4 mm diameter) from 4-week-old *Arabidopsis Col-0* and *cer1-2* plants were collected to measure ROS. *M. truncatula* primary roots growing on 1.5% water agar for 5 days were cut into 0.5 mm strips and incubated in 200 μl water in a 96-well plate (Greiner Bio-one) overnight. For the SmLCO inhibition assay, the infection zone area of wild type and mutant primary roots were used and root segments were pretreated with either water or 10−8 M SmLCO over different time points before being challenged with 0.4 mg/ml PGN or 10−8 M CO4 and/or 10−8 M SmLCO. For cycloheximide treatments, *M. truncatula* roots were pre-incubated with 100 μM cycloheximide (*Sigma-Alrich*) for 2 h. *M. truncatula* root segments were pretreated with 0.1% DMSO or 1 mM Acetazolamide for 30 min before addition of elicitors. After incubation, the water was removed from each well and exchanged with 200 μl reaction buffer containing 10−6 M CO4 or CO8 with 1 mM Acetazolamide, 10 μg/ml horseradish peroxidase (Sigma-Alrich) and 20 μM lumen (Sigma-Alrich) or 0.1 M Tris-Cl buffer (pH 7.5). According to experiments performed. The fungal germinated spores exudates (GSE, 10 times concentrated) were used to detect ROS production in Arabidopsis leaves and
M. truncatula roots. Luminescence was recorded with a Variscan™ Flash Multi-mode Reader (Thermo Fisher Scientific) at indicated time points.

MAPK activity assay. One-week old M. truncatula roots grown on modified Fahraeus medium were excised into small segments and incubated overnight in water. The 1% samples were then treated with 10-4 M CO8 or DMSO for 10 min, then frozen in liquid nitrogen immediately for protein extraction. The SmLCO inhibition assay was performed at the same treatment as detection of ROS production, roots were incubated with water or SmLCO for 30 min, and then treated with 10-4 M CO8 and/or 10-4 M SmLCO for 10 min. The root protein was homogenized in an extraction buffer containing 20 mM HEPES-KOH (pH 7.5), 150 mM KCl, 1 mM EDTA, 0.5% Triton-X-100, 1 mM DTT, complete protease inhibitors (Roche) and phosphatase inhibitors (Roche). Equal amounts of total protein were electrophoresed on 10% SDS PAGE. An anti-pERK antibody (Cell Signalling Technology, Cat#4370, 1:3000 dilution) was used to determine phospho-protein levels. The signals were detected using Pico stable ECL solution (Millipore). Supershift assay was performed using the oligo (ethylene glycol) linker (OEG-linker) to form an oxime linkage at the reducing end. The resulting conjugate containing a free thiol was used in a reaction with a biotin moiety. The biotinylated CO4 and CO8 were then immobilized to streptavidin-coated beads and stored at 25 µM concentration in 50% acetonitrile/water at 4 °C. Conjugates were immobilized on Dynabeads® MyOne™ Streptavidin C1 beads (LifeTechnologies) as follows. Beads were washed three times with PBS, pH 7.2, 0.01% Tween-20 buffer by using a DynaMag®-2 magnet. After washing, the biotinylated ligand was diluted in PBS, pH 7.2, 0.01% Tween-20 buffer and added to Dynabeads® MyOne™ Streptavidin C1 beads. The mixture was incubated for 30 min at room temperature with rotation. After washing three times with PBS pH 7.2, 0.01% Tween-20 and subsequently reconstituted in PBS pH 7.2, 0.01% Tween-20 to a final concentration of 10 mg/ml. After 12 h, the beads were washed three times by lysis buffer and then incubated with the extracted proteins for 1 h. The Dynabeads® MyOne™ Streptavidin C1 beads without crosslinked COs were used as a control. For the binding assay, 50 µl PGN (0.8 mg/ml) was centrifuged to isolate a soluble fraction in the supernatant and an insoluble pellet fraction. The PGN pellet was then mixed with extracted proteins and allowed to bind for 1 h. After washing six times with cold lysis buffer (50 mM HEPES-KOH pH 7.5, 40 mM KCl, 1 mM EDTA, 0.5% Triton-X-100, 1 mM DTT, complete protease inhibitors), the bound protein was recovered by boiling either beads or PGN pellet with SDS loading buffer. For elction competition assays, equal levels of proteins were incubated with CO4 and CO8 beads or PGN pellet and following the wash steps, the bound proteins was eluted with 300 mM NaCl. The supernatant (PF4000) was analyzed for the intensity of CO8 at indicated time points. The anti-HA antibody (Sigma-Aldrich, Cat#12013819001, 1:3000 dilution) was used to detect the protein bound to COs beads and PGN pellet.

Cell death assay in tobacco leaves. Agrobacterium GV3101 transformants carrying the respective construct were diluted to OD600 = 0.6 to infiltrate into fully expanded leaves of N. benthamiana as described above. The plants were then covered in a transparent dome to maintain high humidity and cell death induction was recorded between 24 and 72 h. After 12 h post-infiltration, leaf punches from each infiltrated area were collected to extract total protein. The expression level of MtCO8 beads or PGN pellet and following the wash steps, the bound proteins was eluted with 300 L 2,5-dihydroxybenzoic acid (10 mg/μl) and then treated with either 0.1% DMSO or 1 mM Acetazolamide for 30 min before incubations with CO8. A microtitre sample of from the incubation solution was taken with 2.5-dihydroxybenzoic acid matrix to detect the intensity of CO8 at indicated time points.

Data availability
The RNA-seq data have been deposited in GenBank with accession number SRR097705 [https://www.ncbi.nlm.nih.gov/bioproject/PRJA363004]. Data and materials supporting the observations in this study are available from the authors upon request.

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Author contributions

F.F., J.H.S. and G.E.D.O. conceived and designed the experiments. F.F. and J.H.S. performed the research. T.L. and G.V.R. analyzed RNA-seq data. S.F. synthesized CO and LCO molecules. K.G., M.B.T. and K.R.A. prepared CO4 and CO8 beads. A.G. supported the work on *P. palmivora*. F.F. and G.E.D.O. wrote the paper with comments from Z.B., J.S. and S.R.

Competing interests

The authors declare no competing interests.

Additional information

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