

## eXtra Botany

Insight

# Maintaining osmotic balance in legume nodules

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This article comments on:

**Tian L, Liu L, Xu S, Deng R, Wu P, Jiang H, Wu G, Chen Y. 2022. A D-pinitol transporter, LjPLT11, regulates plant growth and nodule development in *Lotus japonicus*. Journal of Experimental Botany 73, 351–365.**

**Pinitol (3-O-methyl-D-chiro-inositol) is shown to be transported passively across the plant-derived symbiosome membrane in *Lotus japonicus* by the energy-independent polyol transporter LjPLT11. Accumulation of pinitol is crucial to maintenance of osmotic balance in nodules. RNAi lines reduced in LjPLT11 have disrupted symbiosome membranes, reduced N<sub>2</sub> fixation by rhizobial bacteroids and reduced plant growth. There is also an increase in production of reactive oxygen species (ROS) in LjPLT11 RNAi lines, consistent with osmotic imbalance causing increased stress in nodules.**

Biological nitrogen fixation provides 50–70 Tg of bioavailable nitrogen in agricultural systems per year, and sustains global food security. The most efficient contribution to biologically fixed nitrogen is from symbioses between legumes and rhizobia (Herridge *et al.*, 2008), which are soil bacteria that induce formation of nodules on plant roots. Legumes initiate nodule formation by the release of flavonoids, which induce *nod* genes in rhizobia. In turn, the bacteria synthesize lipo-chito-oligosaccharides (LCOs), that are detected by plant LysM-type receptors. This leads to activation of the common Sym pathway that is shared with the more ancient mycorrhizal symbiosis (Oldroyd and Downie, 2004). Root hairs curl, entrapping rhizobia in an infection pocket. Plants form an infection tube which bacteria enter and move down, while remaining extracellular to host cells. At the same time as infection threads develop, nodule development proceeds in the plant cortex, with the developing nodule meeting the dividing and ramifying infections threads. It has recently been shown that lateral roots and nodules

have overlapping developmental programmes, with *Nodule INception* activator (*NIN*) initiating the same programme and sharing downstream activators with the lateral root programme (Schiessl *et al.*, 2019; Soyano *et al.*, 2019). Eventually, rhizobia are endocytosed into the cytoplasm of nodule cells, where the N<sub>2</sub>-fixing form of rhizobia (bacteroids) develop and are surrounded by the bacterial cell- and plant-derived symbiosome membranes. Together, the plant membrane and bacteroids are known as symbiosomes and the space between the two membranes is the symbiosome space. Nodules are either determinate, as in beans, soybeans, and *L. japonicus*, or indeterminate, as in alfalfa, *Medicago truncatula*, pea, and clover. Determinate nodules have a transient meristem resulting in infected plant cells being at the same development stage, with nodules growing larger by cell expansion. Typically, these nodules have several bacteroids enclosed by a single symbiosome membrane all in the same developmental state. Indeterminate nodules have a persistent meristem with distinct development zones from the tip, which is distal to the root, to the base, which is proximal to the root. Indeterminate nodules usually have symbiosomes with a single bacteroid (Oldroyd *et al.*, 2011; Oldroyd and Downie, 2008; Poole and Udvardi, 2013). Distal zone I contains the nodule meristem, zone II branching infection threads, zone II/III interzone developing bacteroids, zone III mature N<sub>2</sub>-fixing symbiosomes, and zone IV senescing symbiosomes.

As bacteroids develop, large changes occur in the transcriptome and proteome, with N<sub>2</sub> fixation genes induced, but most genes required for growth, including ribosomal proteins, DNA replication, and amino acid biosynthesis, having reduced transcription (Barnett *et al.*, 2004; Becker *et al.*, 2004; Karunakaran *et al.*, 2009; Pessi *et al.*, 2007). Some legumes, such as those in a phylogenetic group known as the Inverted Repeat-Lacking Clade (IRLC legumes) produce from seven to >700 nodule cysteine-rich (NCR) peptides (Mergaert *et al.*, 2003; Guefrachi *et al.*, 2014; Montiel *et al.*, 2017; Roy *et al.*, 2020). These can be found in many common agriculturally important legumes such as pea, although most work has been done on the model indeterminate

legume *Medicago truncatula*, where the peptides control bacteroid development. The model determinate legume *L. japonicus* does not produce NCR peptides, so while they exert precise control of bacteroid development, resulting in profound changes in bacteroid physiology and possibly fixation efficiency, they are not essential for the development of an effective symbiosis.

The symbiosome is the effective  $N_2$ -fixing engine of symbiosis, with the plant providing carbon in the form of dicarboxylic acids (mainly L-malate and succinate) as well as metals, and even a key ligand for the  $N_2$ -reducing bacterial enzyme nitrogenase in the form of homocitrate to bacteroids. All nutrients have to traverse two membranes, the first of which is the plant-derived symbiosome membrane (also called the peribacteroid membrane), which is effectively inverted so that movement from the plant cytosol to the symbiosome space equates to export. Metabolites can accumulate in the symbiosome space depending on whether they are then transported across the bacteroid membrane and utilized in metabolism, or not. The main carbon sources, the dicarboxylic acids, are catabolized by the tricarboxylic acid (TCA) cycle. Recent modelling and  $^{13}C$  metabolic flux analysis showed that catabolism of dicarboxylates requires more oxygen but also produces a high ( $NADH/NAD^+$ ) ratio suited to  $N_2$  reduction. While a low  $O_2$  level is required to protect nitrogenase from inactivation, plants limit oxygen supply to bacteroids so as to restrict the decarboxylating arm of the TCA cycle, which limits ammonia assimilation into glutamate. Plants control  $O_2$  levels in nodules with an  $O_2$  diffusion barrier and the synthesis of  $O_2$  binding haem proteins, the leghaemoglobins (Ott *et al.*, 2005). Such a tight control of oxygen supply by legumes while providing dicarboxylates as the energy and electron source donors for  $N_2$  fixation, with their high  $O_2$  requirement for metabolism, promotes ammonia secretion rather than assimilation into the central amino acid glutamate by bacteroids (Schulte *et al.*, 2021). However, modelling, in agreement with experimental studies, also shows that as the  $O_2$  supply becomes even more limited, alanine as well as ammonia will be secreted by bacteroids. This is because alanine synthesis removes pyruvate and reductant ( $NADH$ ) from the bacteroid and prevents further oxidative stress due to  $NADH$  synthesis by the TCA cycle.

While oxygen is clearly the limiting nutrient that controls much of nodule metabolism, osmotic balance is clearly critical to  $N_2$  fixation in nodules. Synthesis of cyclic  $\beta$ -glucan, which seems to balance osmotic potential in the bacterial periplasm (the space between the inner and outer bacterial membranes) by nodule bacteria is essential for effective  $N_2$ -fixing nodules (Bhagwat *et al.*, 1999). In the study reported by Tian *et al.* (2021) in this issue of JXB, a further key player in the maintenance of osmotic balance in the symbiosome space of *L. japonicus* nodules has been identified as the methylated polyol, pinitol. Some 13 polyols have been identified in higher plants and they are important antioxidants and osmoprotectants that play key roles in abiotic and biotic stress resistance (Stoop *et al.*, 1996). In *L. japonicus*, the polyols mannitol, pinitol, ononitol (4-O-methylmyo-inositol), threitol, and sorbitol accumulate in nodules

(Colebatch *et al.*, 2004; Desbrosses *et al.*, 2005). The production of the methylated derivatives of myo-inositol, either as pinitol or as ononitol, or both by legumes is widespread. Peas produce mainly ononitol and this is probably why some *Rhizobium leguminosarum* strains, as well as *Ensifer meliloti*, produce rhizopines such as 3-OMSI (3-O-methyl-scylo-inosamine), by oxidizing and then aminating ononitol on the 3-O group (Geddes *et al.*, 2019). The synthesis of 3-OMSI is under control of the bacteroid-expressed master regulator NifA, and it seems likely that this is then catabolized by sibling rhizobia, either in infection threads or in the rhizosphere. However, the reason for the abundance of pinitol and ononitol in legumes nodules has not been clear until the current work (Tian *et al.*, 2021) which reveals that they are critical to osmotic balance. The authors show by functional analysis in yeast of LjPLT11 from the *L. japonicus*-*Mesorhizobium* symbiosis that LjPLT11 is an energy-independent transporter for xylitol, two O-methyl inositols (pinitol and ononitol), xylose, and galactose. LjPLT11 was shown by immunogold analysis to be located on the symbiosome membrane, where it is predicted to facilitate transport of D-pinitol into the symbiosome space. Knockdown of LjPLT11 by RNAi in *L. japonicus* inhibited plant growth under symbiotic  $N_2$ -fixing conditions and resulted in the formation of abnormal bacteroids with reduced nitrogenase. Strangely, it accelerated plant growth under nitrogen sufficiency, suggesting that synthesis and movement of pinitol has a cost under non-nodulating nitrogen-replete conditions. Interestingly, while LjPLT11 is highly expressed in nodules, it is also expressed in tissues of roots and stems, suggesting that it may have other roles in plant osmotic balance. As predicted from the location of LjPLT11 on the symbiosome membrane, nodules had an increased osmotic pressure in the cytosol and a decreased osmotic pressure in bacteroids particularly 4 weeks after inoculation with *M. loti*. As the symbiosome space is between the bacterial and plant-derived symbiosome membranes, maintenance of the correct osmotic balance would be critical for nutrient exchange, both for uptake of carbon and ions by bacteroids and for secretion of ammonia and alanine to the plant cytosol. It is not clear whether bacteroids themselves accumulate pinitol; however, disruption of the osmotic potential in the symbiosome space is likely to have a knock-on effect on bacteroids. Notably, rhizobia possess their own ways to cope with hyperosmotic conditions. Biosynthesis of compatible solutes such as trehalose or N-acetylglutaminylglutamine amide (NAGGN) contributes to adaptation to high osmolarity environments in *Sinorhizobium* (Flechar *et al.*, 2010; Sagot *et al.*, 2010), *Rhizobium* (McIntyre *et al.*, 2007), or *Bradyrhizobium* (Ledermann *et al.*, 2021a). Whilst trehalose biosynthesis is needed in *Bradyrhizobium* for growth in infection threads (Ledermann *et al.*, 2018, 2021a), no mutants in compatible solute biosynthesis with an effect on bacteroids have so far been reported. This raises the question of how rhizobia adapt to the osmotic conditions in nodules. Bacteroids display a streamlined physiology for  $N_2$  fixation (Ledermann *et al.*, 2021b) and rely on their plant hosts not only for nutrients but also for functions they are able to fulfil as free-living bacteria such as branched-chain amino acid biosynthesis (Prell *et al.*, 2009). The

osmoprotective role of D-pinitol and potentially other methylated myo-inositol derivatives transported into the peribacteroid space as described in the study by [Tian \*et al.\* \(2021\)](#) thus raises the possibility that bacteroids also surrender osmoregulation to their plant hosts. Consistent with the alteration of the osmotic potential in the symbiosome space, RNAi lines of LjPLT11 had misshapen symbiosome membranes. Subsequently, the accumulation and distribution of ROS in infected cells changed in 4-week-old nodules in LjPLT11i plants. It is proposed that this increase in ROS results from the imbalance in osmotic regulation. Overall, this work shows that pinitol maintains osmotic balance and stabilizes the symbiosome membrane.

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## References

- Barnett MJ, Tolman CJ, Fisher RF, Long SR. 2004. A dual-genome symbiosis chip for coordinate study of signal exchange and development in a prokaryote–host interaction. *Proceedings of the National Academy of Sciences, USA* **101**, 16636–16641.
- Becker A, Berges H, Krol E, *et al.* 2004. Global changes in gene expression in *Sinorhizobium meliloti* 1021 under microoxic and symbiotic conditions. *Molecular Plant-Microbe Interactions* **17**, 292–303.
- Bhagwat AA, Mithofer A, Pfeffer PE, Kraus C, Spickers N, Hotchkiss A, Ebel J, Keister DL. 1999. Further studies of the role of cyclic beta-glucans in symbiosis. An *ndvC* mutant of *Bradyrhizobium japonicum* synthesizes cyclodecakis-(1→3)-beta-glucosyl. *Plant Physiology* **119**, 1057–1064.
- Colebatch G, Desbrosses G, Ott T, Krusell L, Montanari O, Kloska S, Kopka J, Udvardi MK. 2004. Global changes in transcription orchestrate metabolic differentiation during symbiotic nitrogen fixation in *Lotus japonicus*. *The Plant Journal* **39**, 487–512.
- Desbrosses GG, Kopka J, Udvardi MK. 2005. *Lotus japonicus* metabolic profiling. Development of gas chromatography–mass spectrometry resources for the study of plant–microbe interactions. *Plant Physiology* **137**, 1302–1318.
- Flechar M, Fontenelle C, Blanco C, Goude R, Ermel G, Trautwetter A. 2010. RpoE2 of *Sinorhizobium meliloti* is necessary for trehalose synthesis and growth in hyperosmotic media. *Microbiology* **156**, 1708–1718.
- Geddes BA, Paramasivan P, Joffrin A, *et al.* 2019. Engineering transkingdom signalling in plants to control gene expression in rhizosphere bacteria. *Nature Communications* **10**, 3430.
- Guefrachi I, Nagymihaly M, Pislariu CI, Van de Velde W, Ratet P, Mars M, Udvardi MK, Kondorosi E, Mergaert P, Alunni B. 2014. Extreme specificity of NCR gene expression in *Medicago truncatula*. *BMC Genomics* **15**, 712.
- Herridge D, Peoples M, Boddey R. 2008. Global inputs of biological nitrogen fixation in agricultural systems. *Plant and Soil* **311**, 1–18.
- Karunakaran R, Ramachandran VK, Seaman JC, East AK, Moushine B, Mauchline TH, Prell J, Skeffington A, Poole PS. 2009. Transcriptomic analysis of *Rhizobium leguminosarum* b.v. *viciae* in symbiosis with host plants *Pisum sativum* and *Vicia cracca*. *Journal of Bacteriology* **191**, 4002–4014.
- Ledermann R, Bartsch I, Müller B, Wülser J, Fischer HM. 2018. A functional general stress response of *Bradyrhizobium diazoefficiens* is required for early stages of host plant infection. *Molecular Plant-Microbe Interactions* **31**, 537–547.
- Ledermann R, Emmenegger B, Couzigou JM, Zamboni N, Kiefer P, Vorholt JA, Fischer HM. 2021a. *Bradyrhizobium diazoefficiens* requires chemical chaperones to cope with osmotic stress during soybean infection. *MBio* **12**, e00390.
- Ledermann R, Schulte CCM, Poole PS. 2021b. How rhizobia adapt to the nodule environment. *Journal of Bacteriology* **203**, e00539–20.
- McIntyre HJ, Davies H, Hore TA, Miller SH, Dufour JP, Ronson CW. 2007. Trehalose biosynthesis in *Rhizobium leguminosarum* bv. *trifolii* and its role in desiccation tolerance. *Applied and Environmental Microbiology* **73**, 3984–3992.
- Mergaert P, Nikovics K, Kelemen Z, Maunoury N, Vaubert D, Kondorosi A, Kondorosi E. 2003. A novel family in *Medicago truncatula* consisting of more than 300 nodule-specific genes coding for small, secreted polypeptides with conserved cysteine motifs. *Plant Physiology* **132**, 161–173.
- Montiel J, Downie JA, Farkas A, Bihari P, Herczeg R, Bálint B, Mergaert P, Kereszt A, Kondorosi É. 2017. Morphotype of bacteroids in different legumes correlates with the number and type of symbiotic NCR peptides. *Proceedings of the National Academy of Sciences, USA* **114**, 5041–5046.
- Oldroyd GED, Downie JA. 2004. Calcium, kinases and nodulation signalling in legumes. *Nature Reviews. Molecular Cell Biology* **5**, 566–576.
- Oldroyd GED, Downie JA. 2008. Coordinating nodule morphogenesis with rhizobial infection in legumes. *Annual Review of Plant Biology* **59**, 519–546.
- Oldroyd GED, Murray JD, Poole PS, Downie JA. 2011. The rules of engagement in the legume–rhizobial symbiosis. *Annual Review of Genetics* **45**, 119–144.
- Ott T, Udvardi MK, Günther C, Krusell L, Desbrosses G, Vigeolas H, Bock V, Czechowski T, Geigenberger P, Udvardi MK. 2005. Symbiotic leghemoglobins are crucial for nitrogen fixation in legume root nodules but not for general plant growth and development. *Current Biology* **15**, 531–535.
- Pessi G, Ahrens CH, Rehrauer H, Lindemann A, Hauser F, Fischer H-M, Hennecke H. 2007. Genome-wide transcript analysis of *Bradyrhizobium japonicum* bacteroids in soybean root nodules. *Molecular Plant-Microbe Interactions* **20**, 1353–1363.
- Poole PS, Udvardi M. 2013. Transport and metabolism in legume–rhizobia symbioses. *Annual Review of Plant Biology* **64**, 781–805.
- Prell J, White JP, Bourdes A, Bunnewell S, Bongaerts RJ, Poole PS. 2009. Legumes regulate *Rhizobium* bacteroid development and persistence by the supply of branched-chain amino acids. *Proceedings of the National Academy of Sciences, USA* **106**, 12477–12482.
- Roy P, Achom M, Wilkinson H, Lagunas B, Gifford ML. 2020. Symbiotic outcome modified by the diversification from 7 to over 700 nodule-specific cysteine-rich peptides. *Genes* **11**, 348.
- Sagot B, Gaysinski M, Mehiri M, Guigonis JM, Le Rudulier D, Alloing G. 2010. Osmotically induced synthesis of the dipeptide N-acetylglutamylglutamine amide is mediated by a new pathway conserved among bacteria. *Proceedings of the National Academy of Sciences, USA* **107**, 12652–12657.
- Schiessl K, Lilley JLS, Lee T, *et al.* 2019. *NODULE INCEPTION* recruits the lateral root developmental program for symbiotic nodule organogenesis in *Medicago truncatula*. *Current Biology* **29**, 3657–3668.
- Schulte CCM, Borah K, Wheatley RM, *et al.* 2021. Metabolic control of nitrogen fixation in rhizobium–legume symbioses. *Science Advances* **7**, eab2433.
- Soyano T, Shimoda Y, Kawaguchi M, Hayashi M. 2019. A shared gene drives lateral root development and root nodule symbiosis pathways in *Lotus*. *Science* **366**, 1021–1023.
- Stoop JMH, Williamson JD, Pharr DM. 1996. Mannitol metabolism in plants: a method for coping with stress. *Trends in Plant Science* **1**, 139–144.
- Tian L, Liu L, Xu S, Deng R, Wu P, Jiang H, Wu G, Chen Y. 2022. A D-pinitol transporter, LjPLT11, regulates plant growth and nodule development in *Lotus japonicus*. *Journal of Experimental Botany* **73**, 351–365.