*NLP2 regulation of Nitrite Reductase is required for vacuole integrity in N-fixing cells of Medicago truncatula under high nitrate*

Suyu Jiang1, Jinpeng Gao1, Yiting Ruan1, Wenjie Liang1, Yisheng Wang1, Manuel Becana3, Ping Xu4, Jeremy Murray\*12

*\*Correspondence to:* [*jeremy.murray@jic.ac.uk*](mailto:jeremy.murray@jic.ac.uk)*.*

*1 CAS-JIC Centre of Excellence for Plant and Microbial Science (CEPAMS), Centre for Excellence in Molecular Plant Sciences (CEMPS), Shanghai Institute of Plant Physiology and Ecology (SIPPE), Chinese Academy of Sciences, China.*

*2 John Innes Centre, Norwich Research Park, Norwich, NR4 7UH, UK*

*3 Departamento de Biología Vegetal, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas, Avenida Montañana 1005, Zaragoza, 50059, Spain.*

*4 Shanghai Engineering Research Center of Plant Germplasm Resource, College of Life Sciences, Shanghai Normal University, Shanghai 200234, China*

*NIN-Like Protein 2* (*NLP2*) is highly expressed in the N-fixation zone of mature nodules of *Medicago truncatula* where it directly activates the expression of *leghemoglobins* through “double” Nitrate Responsive Elements. Unlike NLP1/4, loss of NLP2 does not affect nitrate suppression of nodulation. However, we found that nodules that develop on *nlp2-1* under high nitrate exhibit abnormal vacuole morphology, which is not seen at lower nitrate concentrations. This phenotype is associated with starch accumulation, and higher expression of starch synthesis genes which specifically occurred at 5.0 mM KNO3. Further investigation revealed decreased Nitrite Reductase (NiR) activity and increased levels of nitrite and nitric oxide (NO). Transcript profiling revealed that *Nitrite Reductase* expression was strongly reduced in *nlp2* nodules at all nitrate concentrations, reduced expression of genes important for hypoxia adaptation, such as *Alcohol Dehydrogenase*, *Pyruvate Decarboxylase (PDC),* and increased expression of *S-Nitrosoglutathione Reductase* (*GSNOR*) which is involved in NO metabolism. This was associated with lower levels of ATP and a higher NAD+/NADH ratio, suggesting that energy metabolism is specifically compromised in *nlp2* nodules at high nitrate. Transgenic expression of *NiR* in nodules of *nlp2-1* mutants grown at 5.0 mM KNO3 rescued the vacuole phenotype and restored the expression of *GSNOR* and *PDC* to normal levels. Overall, our data implicates NLP2 plays in energy maintenance under high nitrate through its regulation of *NiR*.

**Key words:** NIN-Like Protein, Hypoxia, Nitrite Reductase, Nitric Oxide