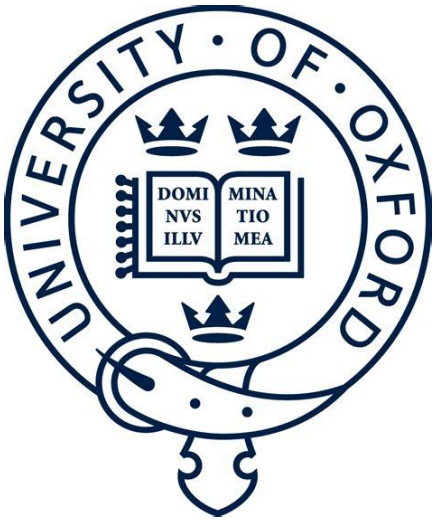
Engineering Control of Rhizobacteria for Plant Growth Promotion



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Declaration of Authorship

I declare that all parts of this thesis are my own work. I would like to acknowledge that in Chapter 3, *A. caulinodans glnB* and *glnK* mutants were made in conjunction with Dr Timothy Haskett. In Chapter 5, IAA plasmids pOPS0666 and pOPS0667, and 2,4-DAPG plasmids pOPS0909 and pOPS0910, were constructed by Dr Barney Geddes. Finally, Δ*nifLA* deletions in *E. radicincitans* and the acetylene reduction assay in figure 5.27 were performed alongside Bergthor Traustason. All contributors mentioned are affiliated with the University of Oxford's Department of Plant Sciences.

This thesis has not been submitted, either partially or in full, for any degree at this university or another institution.

Patrick W.L. Green

Acknowledgements

The world was to me a secret which I desired to divine. Curiosity, earnest research to learn the hidden laws of nature, gladness akin to rapture, as they were unfolded to me, are among the earliest sensations I can remember.

– Mary Shelley

A DPhil is the culmination of years of work, not only my own but also the combined expertise and contributions of so many researchers in this field. None have been more influential on my work than my supervisor Professor Phil Poole. His immense knowledge of complex microbial systems and passion for his work and teaching have been a pleasure to experience during my time at Oxford. The DPhil has not been an easy experience, but his constant support and guidance have kept me on track for which I am very grateful. I will fondly remember the Friday morning discussions on experimental minutiae and rhizobial intricacy, which proved so valuable. I would also like to thank Professor Antonis Papachristodoulou and the Control Engineering group for their discussions and exposure to all things synthetic biology related. I am also extremely grateful for the training and funding I received from the Oxford Interdisciplinary Biosciences BBSRC Doctoral Training Partnership, whose support enabled me to pursue this opportunity.

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Abstract

The engineering of nitrogen fixing bacteria in the soil provides an opportunity to reduce the dependency of agriculture on inorganic fertiliser produced by industry, which leads to ground water pollution and the release of potent greenhouse gases. However, natural diazotrophs lack plant host specificity for root colonisation and employ multilayered regulatory mechanisms that couple the rate of nitrogen fixation with the assimilation of fixed nitrogen, preventing effective release of ammonia to the environment. Native regulation can be overcome by using a synthetic signal from the desired plant host to responsive root colonising bacteria for plant specific control of nitrogen fixation. A trans-kingdom signal was previously developed using bacteria rhizopine molecules allowing specific induction of bacterial gene expression in association with target plant species. Use of this signal to induce bacterial gene expression was tested in the model cereal-associative rhizobium *A. caulinodans*. By engineering bacterial genetic circuitry for rhizopine perception, we were able to improve the sensitivity of rhizopine induced gene expression by 10^3-fold. The rhizopine system was used to demonstrate tight transcriptional control of the NifA master transcriptional regulator of *nif* genes for nitrogen fixation *in vitro*, thereby developing a rhizopine responsive diazotrophic strain which can be assayed for plant growth promotion on rhizopine producing barley lines. To test the feasibility of controlled synthetic-symbiosis in a nodule environment, rhizopine control of nitrogen fixation was assayed in the model symbiotic rhizobium *S. meliloti*. Rhizopine signalling was also amplified via two inducible relay signals, allowing plant dependent control of nitrogen fixation in the cereal associative gammaproteobacterium *E. radicincitans*. This work demonstrates a step towards establishing effective control of nitrogen fixation for plant growth promotion and how the engineering of stringent partner-specific symbiosis could be established in the field for agriculturally relevant cereals.

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List of Abbreviations

Abbreviation	Description
2,4-DAPG	2,4-Diacetylphloroglucanol
3-*O*-MSI	3-*O*-Methyl-s*cyllo*-inosamine
3-oxo-C6-HSL	3-oxo-C6-homoserine lactone
A.U	Arbitrary units
AAA+	ATPase associated
ABA	Abscisic acid
ABC	ATP binding cassette
ACC	L-aminocyclopropane-1-carboxylate
AHL	Acyl-homoserine lactone
AM	Arbuscular mycorrhizal
Amp	Ampicillin
ANOVA	Analysis of variance
ARA	Acetylene reduction assay
aTc	Anhydrous tetracylcine
ATP	Adenosine triphosphate
BNF	Biological nitrogen fixation
dH_2O	Deionised water
DNA	Deoxyribonucleic acid
EBP	Enhance binding protein
EPS	Exopolysaccharide
GC-MS	Gas chromatography mass spectrometry
Gent	Gentamycin
GFP	Green fluorescent protein
GOGAT	Glutamine oxoglutarate aminotransferase
GS	Glutamine synthetase
HCN	Hydrogen cyanide
IAA	Indole acetic acid
IAM	Indole-3-acetimide
IPA	Indole-3-pyruvate
IPTG	Isopropyl β- d-1-thiogalactopyranoside
Kan	Kanamycin
Kb	Kilo-bases
LB	Luria Bertani broth
LCO	Lipo-chitooligosaccharides
Lux	Luciferase
Mb	Mega-bases
MGT	Mean generation time
N	Nitrogen
Neo	Neomycin
NF	Nodulation factor
NFR	Nodulation factor receptor
OD	Optical density
PAS	Per-Arnt-Sim
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGP	Plant growth promoting
QS	Quorum sensing

RBS	Ribosome binding site
RFU	Relative fluorescence units
RLU	Relative luminescence units
RNA	Ribonucleic acid
SEM	Standard error of mean
SI	*Scyllo*-inosamine
SOC	Superoptimal medium with catabolic repressor
SNP	single-nucleotide polymorphism
Strep	Streptinomycin
T7 RNAP	T7 RNA Polymerase
Tet	Tetracycline
Trp	Tryptophan
UMS	Universal minimal salts
WT	Wild type

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Chapter 1

Introduction

1.1 The Role of Nitrogen in Agriculture

Nitrogen is a vital nutrient for crop plants as it is found in amino acids required for plant growth and development as well as in enzymes required to carry out biochemical processes in the plant[1]. Nitrogen forms part of chlorophyll and photosynthetic machinery in the leaves and stems of plants which play an indispensable role in the photosynthetic process of fixing atmospheric carbon into sugars and starch for the plant[2]. Lack of nitrogen availability leads to a yellowing of the aerial parts of a plant called chlorosis, hindering plant development and productivity[3]. Although nitrogen constitutes 78% of atmospheric gas, it is unavailable for direct use by plants[4]. Therefore, plants must access their nitrogen from sources in the soil, whereby plant roots take up reactive nitrogen species such as ammonia (NH_3), ammonium (NH_4^+), or nitrates (NO_3^-)[5]. Historically, the challenge of nitrogen availability has been met by agricultural practices such as crop rotation – growing different crops in the same area across growing seasons to balance the nutrient use to avoid depletion and maintain soil fertility[5,6]. This method has been achieved most successfully by rotating high-value crops, such as cereals, with legume crops which act as 'green manure'[7]. Plants like alfalfa (*Medicago sativa*), white clover (*Trifolium repens*), and vetch (*Vicia sativa*) are cultivated before the biomass is incorporated into the soil, often with other organic matter such as manure[8]. This method is effective for improving soil nitrogen content due to plants, primarily from the *Fabaceae* family, having evolved to form a symbiotic relationship with nitrogen-fixing bacteria which inhabit small growth structures called nodules on the roots of host plants[9]. These bacteria, called rhizobia, are capable of fixing atmospheric nitrogen into biologically available ammonia for the host plant, making them rich in protein content[10]. Incorporating the legume biomaterial into the field allows soil bacteria to convert the abundant amino acids into nitrate (NO_3^-), alongside other micronutrients such as potassium and phosphorus, which are then available for subsequent crop

growth, thereby acting as a fertilizer[11]. Despite the effectiveness of this strategy for millennia, it is costly in terms of time and resources, as well as reducing the period of growth available for more desired crops such as wheat (*Triticum aestivum*), rice (*Oryza sativa*), and maize (*Zea mays*)[12-14].

To meet the demands of modern agriculture, chemical fertilisers were introduced which are produced using the Haber-Bosch process, identified at the start of the 20th century[15]. This reaction directly synthesises ammonia from atmospheric nitrogen by reaction with hydrogen in the presence of an iron catalyst at high temperatures and pressures (500°C, 20 MPa)[15]. The Haber-Bosch process was heralded as a technological revolution and was described as 'bread from air', and allowed for agriculture to keep pace with global population expansion[6]. However, the vast amounts of nitrogen fixed by the process has greatly contributed to a build-up of reactive nitrogen in the biosphere, leading to disruption of the global nitrogen cycle consisting of nitrogen fixation, assimilation, mineralisation, nitrification, and denitrification[3]. The high energy intensity required by the reaction also consumes 1-2% of global energy supply and a significant portion of natural gas supplies[16]. However, the problems are not just with the chemical process but with the overuse of fertiliser to match agricultural demand, which has an encompassing impact on the environment (*Figure 1.1*).

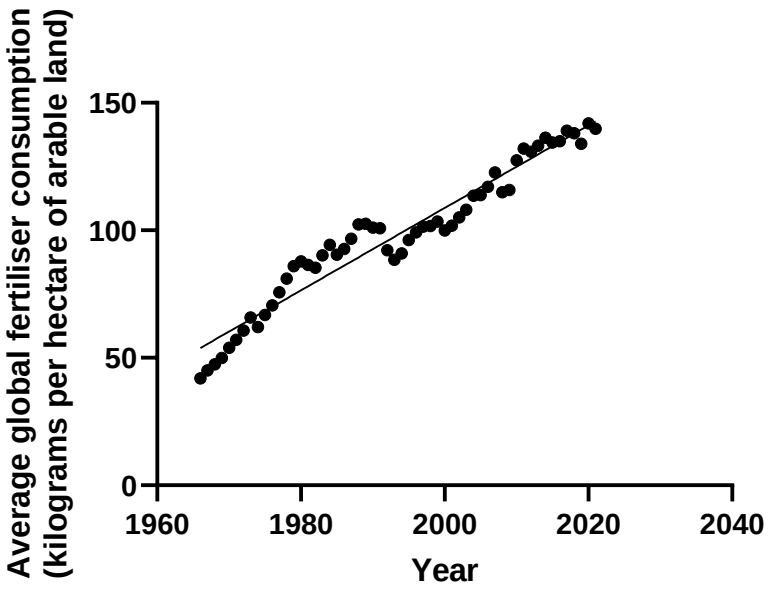


Figure 1.1 Global fertiliser use per hectare of arable land, 1966-2021. Fertiliser covers nitrogenous, potash, and phosphate fertilisers. Arable land includes land defined by the FAO as land under temporary crops temporary meadows for mowing or for pasture, land under market or kitchen gardens, and temporarily fallow land. (Data source: Food and Agriculture Organization of the United Nations via the World Bank).

The nitrogen use efficiency is a product of the crop nitrogen uptake efficiency and nitrogen utilisation efficiency of the fertiliser, and is typically below 50%[8,17]. Nitrogen losses from fertiliser are too high due to poor application methods, leading to much of the reactive nitrogen polluting the environment from agricultural run-off[8,18]. Fertilizer run-off causes eutrophication of larger bodies of water, resulting in an anoxic environment which disrupts the marine ecosystem. Additionally, the nitrification of ammonia to nitric oxide (NO_2) by soil bacteria is a major contributor to greenhouse gas emissions[19]. Since the use of industrial fertilisers, atmospheric nitrous oxide levels have increased by 20% leading to it becoming the third most potent contributor to climate change due to trapping thermal emissions as well as the destruction of stratospheric ozone[19]. However, it is not just the environmental impact of fertilisers which pose a problem, fertilisers contribute significantly to global food security but

often remain economically inaccessible to developing countries due to rising prices driven by consumption in North American and Asian markets. In particular, the projected fertiliser demand in developing countries, such as sub-Saharan Africa, are expected to fall short of the quantities needed to meet goals for future food security (*Figure 1.2*)[20]. Therefore, despite the significant contribution of synthetic fertilisers, there is an increasing interest in developing sustainable biological fertilisers for increased nitrogen use efficiency and to meet the nitrogen requirements for food production in growing demographics.

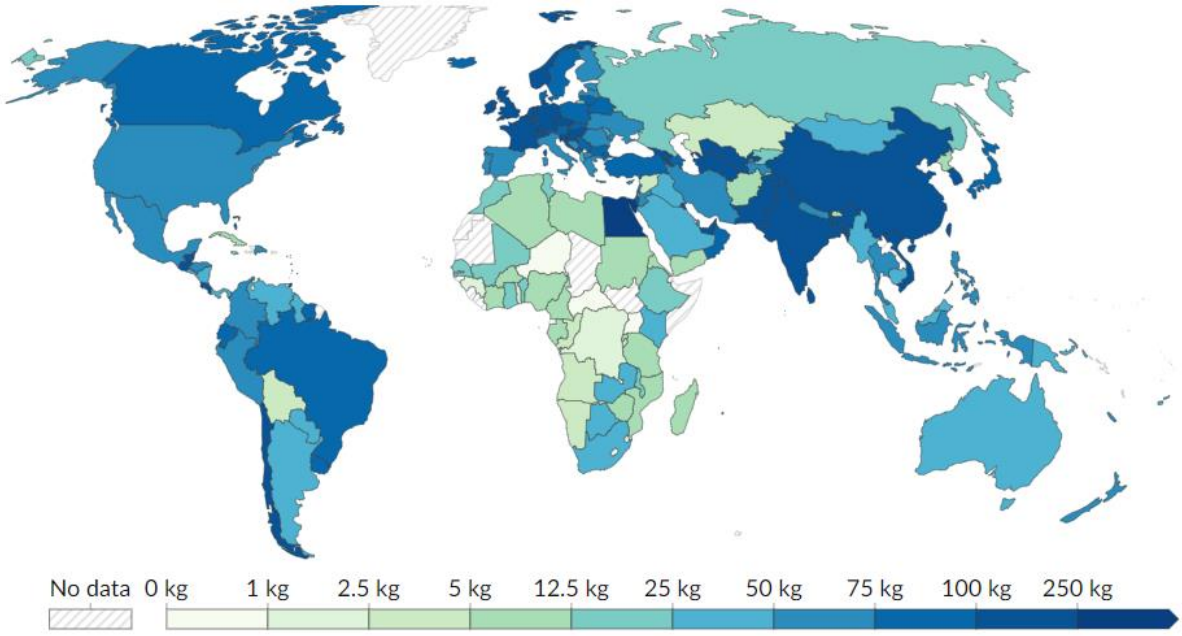


Figure 1.2 Global application of nitrogen fertiliser per country, measured in kilograms of total nutrient per hectare of cropland. Adapted from Hannah Ritchie, Max Roser and Pablo Rosado, 2022 - "Fertilizers". Published online at OurWorldInData.org[21].

1.2 Biological Nitrogen Fixation

Biological nitrogen fixation (BNF) already contributes up to 60% of the fixed nitrogen on Earth[22]. Optimisation of this process will help meet the demands for productivity without

compromising soil health and agricultural sustainability. There is already significant scientific interest in the diversity of nitrogen fixing bacteria, the mechanisms of N_2 fixation and how it can be manipulated, and the selection of microorganisms and soil communities as biofertilisers[23-28]. A comprehensive understanding of the mechanisms underpinning BNF may facilitate the transfer of N_2 fixation to other microorganisms or non-leguminous high-value crops. BNF is limited to prokaryotes which are called diazotrophs, which exhibit considerable biodiversity as BNF is found in most phylogenetic groups[29].

Diazotrophs can be classified into three groups: bacteria capable of free living N_2 fixation (*K. pneumoniae*, *A. vinelandii*, *Paenibacillus* sp), associative N_2-fixers (*Nostoc, Azospirillum. brasilense*), and obligate-symbionts (*Rhizobium meliloti*, *Bradyrhizobium japonicum*, *R. leguminosarum, Frankia*)[30,31]. The most studied example of BNF for plants is the symbiotic relationship of *Rhizobia* species with legumes. Symbiotic nitrogen fixation is a form of a mutualistic relationship in which plants provide bacteria with an anaerobic niche and a supply of fixed carbon, due to the high energetic demands of BNF, in exchange for fixed nitrogen provided to the plant host[32]. In agriculture, endosymbiotic N_2 fixation is seen in the close symbiotic relationship between bacterial rhizobial species and legume hosts leading to the efficient assimilation of nitrogen by legumes for maximal growth and development[10,33]. The establishment and functioning of an effective symbiosis between a legume and cognate *Rhizobia* is dependent on genetic determinants in both plant and bacteria which allow for partner recognition, penetration, stimulation of host cell division, and differentiation of the endosymbiont[34]. Alternative forms of biological nitrogen fixation seen in *Rhizobia* are associative nitrogen fixation and free living nitrogen fixation. Plant associative diazotrophs consists of epiphytic bacteria which form micro-colonies or biofilms on the root surface. There are also rhizobial endophytes which enter the roots via crack entry but only form a loose mutualism with host plants. Associative diazotrophs are characterised by more stringent

regulation of N_2 fixation and less efficient delivery of ammonia to host plants[11,35]. Free living nitrogen fixation is performed by bacteria in the rhizosphere without direct interaction with other organisms to provide nitrogen for their own growth[36].

In all diazotrophs conversion of atmospheric N_2 to NH_3 is catalysed by a nitrogenase enzyme complex[37]. To date, three distinct types of nitrogenase have been characterised, differing in the metallic co-factor employed by the enzyme: the MoFe-nitrogenase, the VFe-nitrogenase, and the Fe only-nitrogenase[30,38]. Despite the different metal catalysts used, the enzyme complexes are structurally and mechanistically related[39]. The first identified and the best studied is the Molybdenum (Mo) nitrogenase, due to its ubiquity amongst diazotrophic species[40]. In the case of the iron-molybdenum metalloenzyme complex, the first iron protein called dinitrogenase reductase, which is a homodimer of NifH, functions as an electron donor to a larger $\alpha_2\beta_2$ heterotetrameric dinitrogenase, consisting of NifD α-subunits and NifK β-subunits (*Figure 1.3*)[41]. The dinitrogenase protein consists of a 7Fe-8S P cluster which associates and transfers electrons to the FeMo-cofactor at the enzymatic active site (*Figure 1.3*)[42]. The inventory of genes, encoded in *nif* clusters, required for N_2 fixation alongside nitrogenase varies amongst bacteria and is dependent upon the environmental niche the bacterium occupies and host physiology. Alongside *nifH*, *nifD*, *nifK*, the genes *nifE*, *nifN*, and *nifB* are the minimal required for the synthesis and assembly of the catalytic FeMo-cofactor[43,44]. Additional genes in the *nif* operon encode proteins involved in electron transport to nitrogenase, regulation, and some proteins with unknown functions[43]. Due to the electron transport requirements of the nitrogenase metal-clusters, the enzyme is intolerant of oxygen, therefore many diazotrophs possess additional operons involved in electron transport and oxygen regulation to maintain anoxic conditions[45]. Similarly, nitrogen fixation is a highly energy-intensive process, requiring a ratio of 16 ATP molecules to reduce 1 molecule of N_2[32]. Many symbiotic rhizobia encode additional operons such as *fixABCX* which have been shown

to ferry electrons between nitrogenase and other cellular processes such as respiration, as well as *fixLJK,* which regulate *nif* genes in response to cellular oxygen concentrations, thereby balancing the oxygen and energy requirements of a diazotroph[46,47].

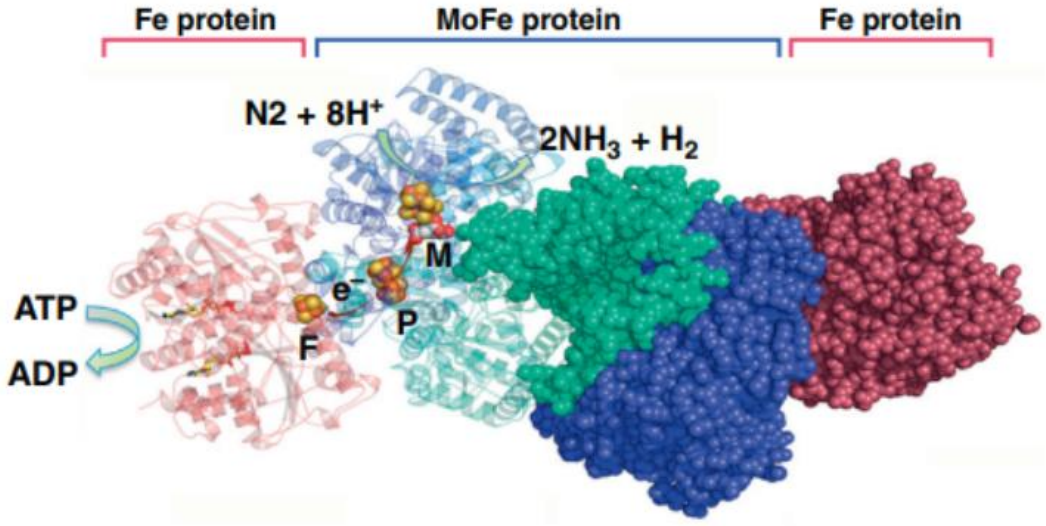


Figure 1.3 Structure of the nitrogenase enzyme complex containing the MoFe and Fe protein components. The three metalloclusters are shown in the complex as the [4Fe4S] abbreviated as F, P cluster as P, and MoFe-co as M, respectively. ATP is hydrolysed by the Fe protein and the electron, shown as e-, is transferred via the metalloclusters to the catalytic site for the conversion of N_2 to NH_3. Adapted from Oldroyd and Dixon, 2014[48].

1.2.1 Symbiotic Nitrogen Fixation

The most complex form of diazotrophic plant-microbe association is endosymbiosis. Diazotrophic bacterial partners are recruited from the environment and accommodated within specific structures inside plant cells[49]. For example, *Nostoc* cyanobacteria are signalled to *Gunnera* seedlings by the secretion of carbohydrate-rich mucilage whereupon they are hosted in the inner cortex of plant cells and exchange nutrients via the host's plasma membrane[33]. Legume-*Rhizobium* symbioses are initiated by the secretion of flavonoid molecules into the surrounding rhizosphere[50]. The flavonoids are taken up by cognate rhizobia whereupon they

bind the NodD transcriptional regulator activating downstream nodulation (*nod*) genes[51]. In response to plant-derived flavonoids, rhizobia synthesize Nod factors (NFs) which, at very low nanomolar concentrations, elicit a symbiotic response in the roots of legumes by binding nod factor receptors (NFRs)[52-54]. NFs have been characterized in several rhizobia, they all constitute lipo-chitooligosaccharides (LCOs) with acetylglucosamine backbones and fatty acyl chains[55,56]. The length of the N-acyl chain and additional modifications such as saturation, glycosylation, and sulfation determine the specificity of the LCO and its perception by the host legume[57]. For example, *Sinorhizobium meliloti* strains that are mutated in the sulfur transferase gene, *nodH*, produce a Nod factor lacking a sulphate group. These mutants are unable to activate any nodulation responses in the host plants *Medicago truncatula*[52].

Nod factor binding of NFRs triggers a complex intracellular signal cascade through the symbiosis signalling pathway, initiated by calcium spiking, which induces symbiotic genes for nodule organogenesis[49,56-60]. Symbiotic gene expression initiates plant cell division and meristem formation at the nodule primordium. The rhizobia infect dividing root cells via crack entry, or by the formation of root hair infection threads, whereby the root hair deforms and forms a pocket entrapping the rhizobia (*Figure 1.4*)[34]. Root hair infection is more common and is followed by an infection thread tubular structure composed of plant cell wall components that allow entry of the bacteria into the cortical cells of the plant[54,61]. Rhizobia are released at the basal tip of the infection thread into the host cell cytoplasm in an infection droplet. The rhizobia are enveloped by a plant-derived membrane in root cortical cells, forming the symbiosome[62]. Within the root cell, the bacteria differentiate into nitrogen fixing bacteroids which are hosted inside the plant as an organelle like symbiosome, whereby a plant-derived membrane controls the nutrient exchange between the host and bacteroid (*Figure 1.4*)[63]. In return for fixed nitrogen, the plant typically provides its bacterial symbiont with a dicarboxylic acid carbon source, amino acids, and other micronutrients which the rhizobia depend upon to

sustain metabolism and nitrogen fixation[32]. Once the symbiosome is established, *Rhizobium* bacteroids significantly alter their nitrogen metabolism[64]. Post-translational modification of glutamine synthetase prevents ammonia assimilation and bacteroids become dependent on the plant for the supply of amino acids. The bacterial ammonium transporter AmtB, which transports ammonium into the bacteroid, is repressed in bacteroids ensuring fixed nitrogen is lost from the cell to the host plant[25]. Ammonia released by the bacteroid is pronated to ammonium due to the acidic symbiosomes environment. The ammonium is transported across the symbiosomes membrane into plant cells where it is rapidly assimilated into amino acids by the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway[65,66]. Nodules provide an ideal environment for optimal nitrogenase activity, with the plant host protecting against oxygen species by several mechanisms. Oxygen diffusion into the nodule is restricted by the outer cells and diffusion into bacteroids by external mucilage[67]. Additionally, plant cells produce leghaemoglobin, a protein which contains a haem group which buffers the free oxygen concentration in the cytoplasm of infected plant cells. Leghaemoglobin maintains a free oxygen concentration that is low enough to allow nitrogenase to function, whilst keeping enough total oxygen to allow cellular aerobic respiration[68]. The presence of leghaemoglobin gives nodules their characteristic pink colour. Forming and maintaining nodules is resource intensive. Thus, the host plant tightly regulates the number of nodules it forms in response to the nitrogen status of growing conditions and additional abiotic and biotic factors[58,69]. However, the close association of the plant and its rhizobial diazotrophic partner allows for optimal delivery of fixed nitrogen to the legume host, enabling plants to thrive in nutrient-poor soils.

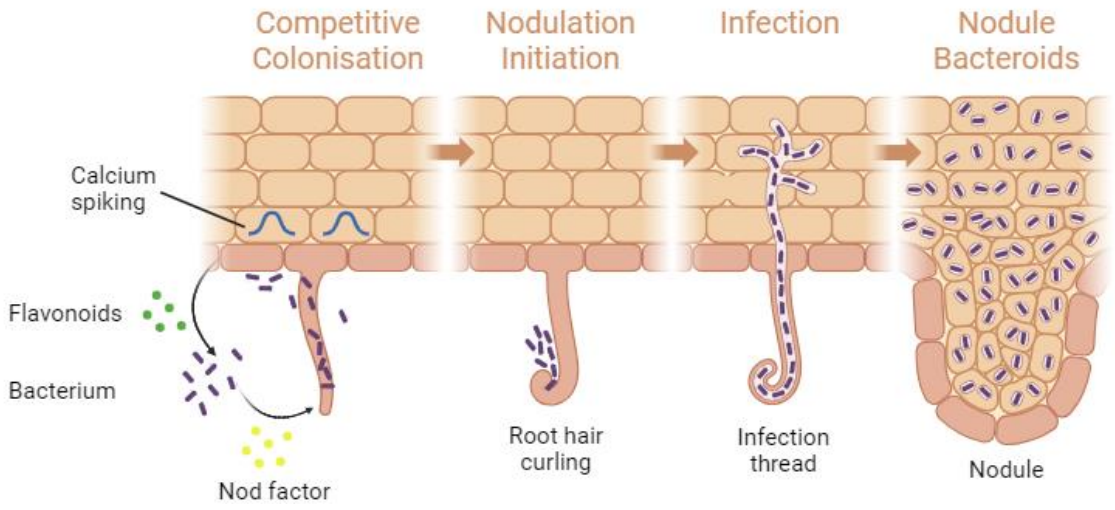


Figure 1.4 Rhizobial colonisation of the root for symbiotic nitrogen fixation inside nodules. The legume root signals to rhizobia by the secretion of flavonoids (green). The rhizobia (purple) in turn produce Nod factors (yellow) that are recognized by the plant. Nod factor perception activates the symbiosis signalling pathway, leading to calcium oscillations, which start in epidermal cells but progress to cortical cells allowing bacterial infection. Rhizobia enter the root by the curling of root hair cells around the bacteria attached at the root surface. Root hair curling is followed by infection threads that are initiated at the site of root hair curls allowing progression of the rhizobia into the root tissue. Nodule organogenesis initiates below the site of bacterial infection via the formation of a nodule meristem in the root cortex. The infection threads grow towards the emergent nodules where the dividing nodule cells are colonised by the bacteria, allowing the rhizobia to differentiate into a nitrogen fixing state.

1.2.2 *Sinorhizobium meliloti*

A model organism for the study of rhizobia-legume symbiotic nitrogen fixation is the alphaproteobacteria *Sinorhizobium (Ensifer) meliloti*[70]. The rhizobia *S. meliloti* forms symbiosis with legumes of the genera *Medicago* and *Melilotus*, including the forage crop alfalfa (*Medicago sativa*) and the model legume *Medicago truncatula*[57]. *S. meliloti* has a moderate genome size of 6.7 Million base pairs[71]. The genome consists of three replicons: A single chromosome of 3.65 Mega-bases (MB), as well as two megaplasmids pSymB and pSymA which are 1.68 Mb and 1.35 Mb respectively[72-74]. Each replicon carries genes required

for the bacteria's symbiosis with its host legume. The chromosome is responsible for all the housekeeping genes of *S. meliloti* cells, but also carries genes involved in plant interaction, responding to external stress, mobility, and chemotaxis[73]. The larger plasmid, pSymB, carries genes involved in nutrient uptake and attachment and entry of the plant host. The smallest plasmid, pSymA has typically been referred to as the symbiotic plasmid, as it carries *nod* genes required to initiate symbiosis and *nif* and gene clusters, responsible for nitrogen-fixing capabilities of the bacterium[75]. The pSymA plasmid has been a candidate of engineering efforts to reduce the plasmid size to a minimal compliment of 58 genes required for effective nodulation of *Medicago*[75]. *S. meliloti* both interacts with the plant host and undergoes a distinct developmental and metabolic shift into nitrogen-fixing bacteroids[63].

S. meliloti produces Nod factors which contain four glucosamine units, an acyl chain of 16 carbon atoms with two unsaturated bonds (determined by NodE and NodF), the terminal reducing glucosamine residue of the Nod factor is sulphated by NodH, NodP and NodQ, whereas the other terminal glucosamine contains an acetyl group added by NodL[55]. *S. meliloti* Nod factors bind NFP and LYK3 high-affinity cell surface receptors on the roots of *M. truncatula* leading to bacterial infection[54,76]. The specific binding of *M. truncatula* Nod factor receptors leads to the activation of symbiosis signalling which results in expression of the transcription factors NIN and ERN1[49,61]. These in turn regulate gene expression associated with bacterial infection via the nod-box promoter sequence. Induction of early nodulin genes, the activation of the inner cell layers, the establishment of root hair deformation and curling, and the initiation of the infection thread allows bacterial infection of the root[77]. *S. meliloti* also possesses *exo* and *exp* genes which are required for EPS formation during infection. The succinoglycan EPS produced may provide passive protection from plant defence reactions inside the infection thread[78]. *EPS-I* mutants fail to initiate infection thread formation and cannot form nitrogen fixing nodules[79]. As the rhizobia move down the infection thread, cytokinin

signalling and induced transcription factors *NIN* induce the transcriptional network of root cortical cells leading to cell division of the nodule primordium where the rhizobia infect and differentiate into nitrogen fixing bacteroids[34,49,59]. The nodules formed by *S. meliloti* on *Medicago* are described as indeterminate. These nodules are elongated and have a persistent meristem that continually produces new cells which can infected by rhizobia inside the nodule, resulting in a gradient of developmental stages inside the nodule (*Figure 1.5)*[80].

Figure 1.5 Nodules of *S. meliloti* CL150 inoculated on roots of the legume *M. truncatula.*

Inside the nodule, *S. meliloti* differentiates into nitrogen fixing bacteroids which synthesise large quantities of MoFe nitrogenase, up to 20% of total cell protein[46]. The transcription of nitrogenase complex is stringently regulated inside bacteroids, where the respective cellular nitrogen and oxygen status regulatory cascades cohere to regulate *nif* gene expression and nitrogenase activity[81]. The transcriptional activator NifA, together with a specific RNA polymerase sigma factor σ^{54}, regulates the transcription of *nif* genes[82]. The *nifA*

gene is highly conserved across rhizobia[23], and is a member of the enhancer binding protein (EBP) family of transcription factors. NifA contains a conserved domain structure consisting of an N-terminal regulatory domain, a central ATPase domain, and C-terminal DNA binding domain[83]. The DNA binding domain consists of a helix-turn-helix and recognises upstream activator sequences which together with σ^{54}-dependent promoters promote *nif* gene transcription[84]. The N-terminal region of NifA contains a GAF-domain, a regulatory module found in species across all kingdoms of life[85]. GAF domains bind small molecules which affect the activity of neighbouring catalytic domains. Signals binding the GAF module of EBPs regulates the interaction of the AAA+ ATPase domain with the σ^{54} RNA polymerase sigma factor, which promotes open promoter complex formation and gene transcription[84]. Across diazotrophic species, the GAF domain regulatory mechanism varies significantly, and in species like *S. meliloti* is yet to be elucidated[86].

In *S. meliloti* the transcription of *nifA* is controlled by the two-component system FixL-FixJ which induces downstream gene transcription at microoxic levels found in the nodule environment[46]. FixL, a histidine kinase, has multiple predicted transmembrane helices and is bound by the membrane. FixL also contains a PAS domain with a haem prosthetic group[81,87]. Oxygen is able to reversibly bind to the haem group, regulating the kinase activity of FixL[87]. Binding of oxygen deactivates the kinase activity, whilst in the absence of oxygen FixL transfers a phophoryl group to FixJ which undergoes a confirmational change allowing removal of steric inhibition of the transcriptional activator domain whilst promoting dimerization of the protein[81,88]. The FixLJ system controls expression of only a few gene targets such as *fixK* and *nifA* (*Figure 1.6*). The active FixJ dimer binds DNA operator sequence upstream of both *nifA* and *fixK* genes where it recruits an RNA polymerase for transcription[88]. NifA acts as an EBP and interacts with the σ^{54} RNA polymerase sigma factor to regulate gene transcription[84]. The sigma factor σ^{54} interacts with the core RNA polymerase and recognises consensus sequences

at -12 and -24 base pairs upstream of the transcriptional start site of *nif* and *fix* genes[84,89]. However, transcription is dependent on NifA binding an upstream activator sequence with a TGT-N10-ACA consensus sequence[89,90]. The interaction of NifA with the σ^{54}- holoenzyme causes DNA looping and nucleotide hydrolysis which allows an open promoter complex to form and transcription to proceed[84]. The NifA-RpoN transcription factor complex induces expression of *nif* genes for the nitrogenase complex, and *fix*ABCX genes which form the electron transport chain required for nitrogenase function[91].

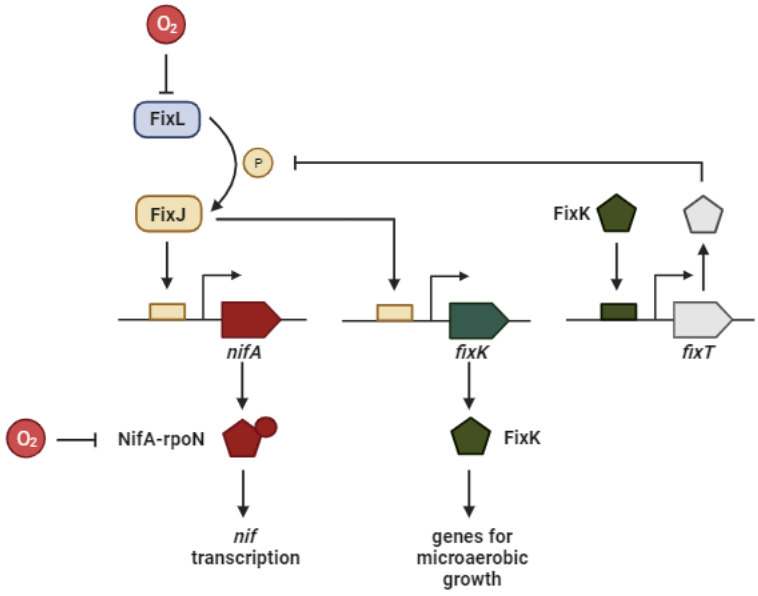


Figure 1.6 Regulatory cascade of *nif* transcription in *S. meliloti*. The oxygen responsive FixL-FixJ two-component system controls expression of *nifA* and *fixK* transcription factors. NifA activity is repressed by oxygen and with RpoN will only induce *nif* genes for nitrogen fixation under anaerobic conditions. FixK regulates expression of *fixNOQP and fixGHIS* required for anaerobic growth, as well as the transcription of *fixT* which represses *fixK* expression forming an autoregulation loop.

After transcriptional activation by FixLJ, FixK regulates expression of the *fixNOQP* and *fixGHIS* operons which encode a terminal oxidase with high oxygen affinity, required for microaerobic growth, and the proteins required for its assembly[92]. FixK also induces expression of *fixT* which acts to inhibit phosphorylation of FixL, thereby forming an autoregulation loop[93].

In symbiotic diazotrophs such as *S. meliloti*, NifA proteins have an additional linker between the ATPase and DNA binding domains, which contains a cysteine repeat motif that regulates NifA at the post-translational level in response to cellular oxygen[82,92]. Therefore, the primary regulator of nitrogen fixation in *S. meliloti* symbiosis with *M. truncatula* is low O_2 tension (*Figure 1.6*), which is critical both for the induction of genes required for nitrogen fixation and the subsequent exchange of nutrients between plants and bacteroids.

1.2.3 Associative Nitrogen Fixation

Nodulation is a highly effective method for the delivery of fixed nitrogen to a host plant, however, it is restricted to plant species within the legume and actinorhizal families[94]. Due to the complexity of the signalling, developmental, and metabolic process required to establish and maintain nodulation, it is a monumental challenge to transfer to cereal crops[27,95]. Whilst cereals cannot form a nodule symbiosis with rhizobia, they can obtain nitrogen from associative diazotrophs[35]. One solution to the nitrogen problem is to engineer diazotrophic bacteria that associate with cereals – whether they are present in the rhizosphere as free-living diazotrophs, on the root surface (epiphytes), or colonising root cells or intercellular spaces (endophytes)[25,62,96]. Bacterial colonisation of the rhizosphere first involves bacterial recognition of primary metabolites from root exudates, leading to cognisant bacteria migrating towards the root surface by chemotaxis[34]. Some soil bacteria have evolved to enter the root tissue via crack entry caused by wounds, or at the emergence points of new lateral root growth[62]. Bacteria then colonise the intercellular spaces in the epidermal and cortical regions and to a lesser extent the vascular tissue[97]. Alternatively, some bacteria associate with cereals as epiphytes and only colonise the surface of roots where they establish microcolonies or biofilms[22]. Several bacteria isolated from these niches have been shown to be diazotrophs[98]. However, their regulation of nitrogen fixation appears to be selfish and thereby deprives their plant host of fixed nitrogen[25,99]. Due to the energetic cost of nitrogen fixation and adapting to

fixation in an aerobic environment, diazotrophic endophytes switch off nitrogen fixation in the presence of ammonia via down-regulation of *nif* genes[46,91].

In some countries, sugarcane is planted with minimal fertilizer use, and there is evidence that the low levels of nitrogen application can be compensated by BNF[24]. An endoyphtic diazotroph *Gluconacetobacter diazotrophicus* Pal5*,* an alphaproteobacteria, has been isolated from the intercellular spaces of the roots, stem, and leaves of sugarcane[100]. *G. diazotrophicus* displayed good acid tolerance and ability to fix nitrogen in media with high ammonium and sugar concentration[101]. Delivery of fixed nitrogen to the host sugarcane has been shown by ^{15}N incorporation and by inoculation of sugarcane with a *nif- G. diazotrophicus* strain, which led to reduced plant growth and lower N content relative to WT strains[102]. Alongside BNF, *G. diazotrophicus* also displays antibacterial and antifungal activity, producing a bacteriocin that inhibits the growth of the sugarcane pathogen *Xanthomonas albilineans* and possesses antifungal activity against *Fusarium sp*[103]. The strain also appears to promote plant growth by solubilising phosphate and by the production of plant hormones via two independent pathways for auxin biosynthesis[100]. Due to the versatility of Pal5, it has also been shown to promote the growth of *Zea mays* under drought and low nitrogen conditions[101].

A key example of a diazotrophic endophyte is *Pseudomonas stutzeri* A1501, isolated from rice roots in China. *P. stutzeri* has a single 49 Kb nitrogen fixation cluster, probably acquired via horizontal transfer, which contains genes that adapt N_2 fixation for appropriate aerobic conditions[104]. *P. stutzeri* is a versatile diazotroph and can switch between denitrification, nitrification, and nitrogen fixation under anaerobic, aerobic, and microaerobic conditions, respectively[105,106]. The strain has been shown to provide excess fixed nitrogen to the host and has therefore been used as a field inoculant for rice and maize crops where it has been shown to promote plant growth through N_2 fixation and other PGP mechanisms[107-109].

Another endophytic coloniser is *Azoarcus sp.* BH72 which colonises kallar grass[110]. *Azoarcus* has been shown to fix nitrogen in the aerenchyma of field-grown kallar grass roots, producing a 60% growth increase in nitrogen-starved conditions in a field trial versus a *fixK* mutant[110]. It is also able to colonise the globally important cereal rice, where it was capable of endophytic N_2 fixation[111]. *Azoarcus* may undergo a lifestyle change upon endophytic colonisation as it becomes unculturable from the roots of kallar grass and rice[110]. However, it colonises roots to great density, Up to 10^8 colony forming units per gram of root fresh weight have been reported[110]. Furthermore, *Azoarcus* appears to be benign as an endophyte, as it lacks type III and IV toxin secretion systems, which transport a variety of effector proteins into the extracellular space or into the cytoplasm of plant host cells thereby affecting the plant-microbe interaction. *Azoarcus* also possesses minimal cellulose-degrading enzymes, and lacks a quorum sensing system, which may contribute to its versatility as an endophyte[112]. However, host plants appear to be selective of endophytic bacteria which colonise them. Land races of rice were preferentially colonised by *Azoarcus,* whilst modern cultivated varieties were preferentially colonised by diverse diazotrophs, such as *Azospirillum* spp., *Klebsiella* sp., and *Burkholderia* sp[113]. Species like *Azoarcus* and *Gluconacetobacter* are tightly associated with their plant host and do not survive well in soil. Bacteria such as *Azospirllum* are found free-living in soil and in close association with cereal roots and display more diverse interactions[114].

Azospirillum brasilense has been well studied for its ability to colonise wheat and rice through the invasion of lateral root cracks, and its ability to promote plant growth through several mechanisms[66,115]. Alongside nitrogen fixation, *A. brasilense* secretes multiple phytohormones, the most common being indole acetic acid (IAA) to stimulate growth of the host plant[116-118]. Phytohormones produced by bacteria regulate root development, enhancing root branching and elongation, which promotes the uptake of soil water and minerals improving host plant growth[119]. *A. brasilense* also carries the gene ACC deaminase (acdS), which reduces

plant ethylene levels and signs of environmental stress[114]. Production of flavonoids has been found in *Azospirillum* strains colonising wheat, where they appear to increase lateral root colonisation. *Azospirillum* inoculation on rice plants even appears to enhance disease resistance against bacterial pathogen *Xanthomonas oryzae*[115]. Due to its productivity in PGP, *Azospirillum* strains have been developed as inoculants for crops in Europe and Africa[35]. It is therefore clear that endophytic interactions are less stringent, with N_2 fixing endophytes able to colonize a broader array of plant hosts. The versatility of promiscuous strains makes them valuable as potential candidates for inoculation of cereals, a key example of this is *Azorhizobium caulinodans*.

1.2.4 *Azorhizobium caulinodans ORS571*

The rhizobia *Azorhizobium caulinodans* is an α-proteobacteria which is capable of symbiosis with the tropical legume *Sesbania rostrata,* forming root nodules through a nod factor dependent mechanism, or producing stem nodules via crack entry (*Figure 1.7*)[120]. *A. caulinodans* is also an endophyte of many plants, such as the cereals *O. sativa* and *T. aestivum* (*Figure 1.7*)[121,122]. *Azorhizobium* is also capable of free living nitrogen fixation under microaerobic conditions and is tolerant of a range of temperatures, from 12 to 44°C, with optimum growth at 37°C[123]. Although ORS571 has been shown to be closely related to rhizobia such as *Bradyrhizobium*, it shows comparatively faster growth characteristics[124]. *A. caulinodans* possesses a single large chromosome of 5.37Mb, which contains the *nif* cluster, encoding genes for nitrogenase function and assembly, synthesis of MoFe cofactor and FeS clusters, and electron transport[125]. ORS571 also encodes genes *hup, hyp* and *hoxA*, encoding proteins for oxidizing the by-product H_2 as an ATP source generated during the nitrogenase reaction[125]. The ORS571 genome also contains a discrete 87.6Kb symbiosis island with nodulation genes (*nodABCSUIJ*), chemotaxis, and type IV secretion system genes[125]. *Azorhizobium* has been extensively studied for its nitrogen regulatory network and its ability

to fix nitrogen in liquid culture, making it an ideal chassis for developing control of nitrogen fixation[126].

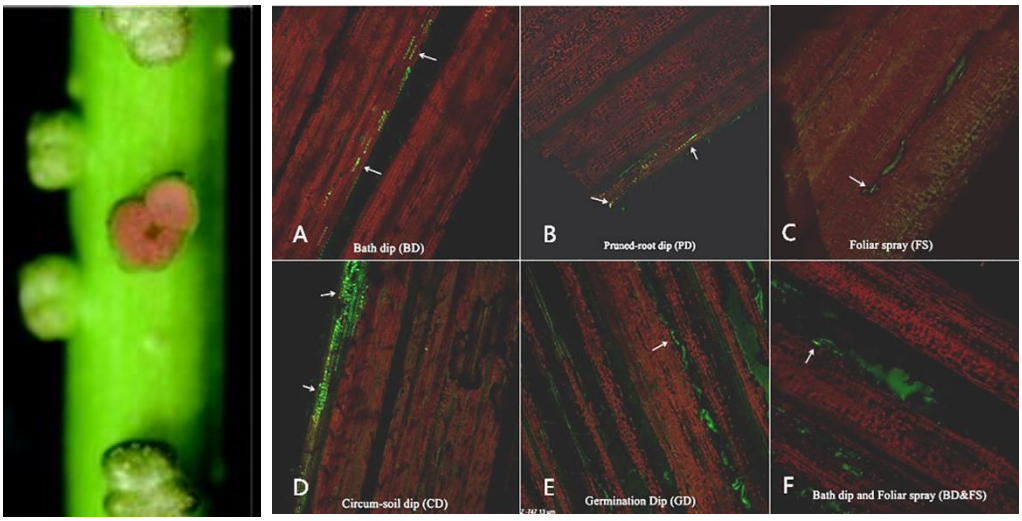


Figure 1.7 Nodules of *A. caulinodans* ORS571 on *Sesbania rostrata* (left), adapted from Suzuki *et al,* 2007[127]. Endophytic establishment of GFP tagged *A. caulinodans* in wheat through the use of different inoculation methods (right), adapted from Liu H *et al*., 2017[128].

Azorhizobium caulinodans ORS571 is unusual for its ability to fix nitrogen as a symbiotic partner, or as an endophyte in cereal roots, and in free living conditions[124]. Due to its versatility and the need to protect nitrogenase from oxygen inactivation under these three conditions, ORS571 has complex regulation of N_2 fixation[129,130]. ORS571 encodes a MoFe dinitrogenase complex and Fe dinitrogenase reductase encoded by *nifHDK*. ORS571 has duplicate *nifH* genes encoding for dinitrogenase reductase, where *nifH1* is mainly active for N_2 fixation in free living conditions and *nifH2* mainly during symbiosis with *Sesbania*[125]. The regulatory network for the expression of *nif* and *fix* genes bears resemblance to *S. meliloti,* however, *nif* regulation is responsive to both cellular nitrogen and oxygen concentrations in free-living conditions[130,131]. In ORS571 Nitrogen-rich conditions repress *nifA* expression

completely, regardless of the oxygen status. Whilst in symbiotic systems, *nif* regulation is primarily from the free-oxygen concentration, as seen in *S. meliloti*[132].

The transcriptional activator NifA, alongside σ^{54} RpoN, controls the expression of *nif* and *fix* genes in both free-living and symbiotic states. Like, *S. meliloti* the regulatory *fixLJ* two component system confers oxygen sensitivity to the transcription of *nifA*. However, unlike symbiotic diazotrophs, FixK rather than FixJ directly activates *nifA* expression[130,131]. A further two nitrogen sensing systems with regulatory proteins NtrBC, NtrYX sense intracellular and extracellular N concentrations and mediate *nifA* expression under nitrogen limiting conditions (*Figure 1.8*)[46,133]. NtrC and NtrX act as transcriptional regulators whilst NtrB/Y function as signal transduction histidine kinases in the two component systems[125]. The cellular nitrogen status is sensed by the GlnD nitrogen regulatory protein, an uridylyltransferase, which transmits the signal via the PII protein (GlnB) to the sensor kinase NtrB. Under nitrogen-limited conditions, NtrB phosphorylates its cognate response regulator NtrC, which in turn activates transcription from the *nifA* promoter[132,134]. Under conditions of nitrogen excess, transcriptional activation by NtrC is prevented via dephosphorylation by the phosphatase activity of NtrB. The NtrC and NtrX proteins share 35.6% sequence homology and both contain a REC domain for phosphorylation and AAA+ ATPase domains for formation of open promoter σ^{54}-RNA polymerase complexes. Signal transduction proteins NtrB and NtrY share 30.8% sequence homology and contain PAS and histidine kinase domains. The NtrY/X two-component regulatory system homologs likely originated from a duplication event of the *ntrBC* locus[133].

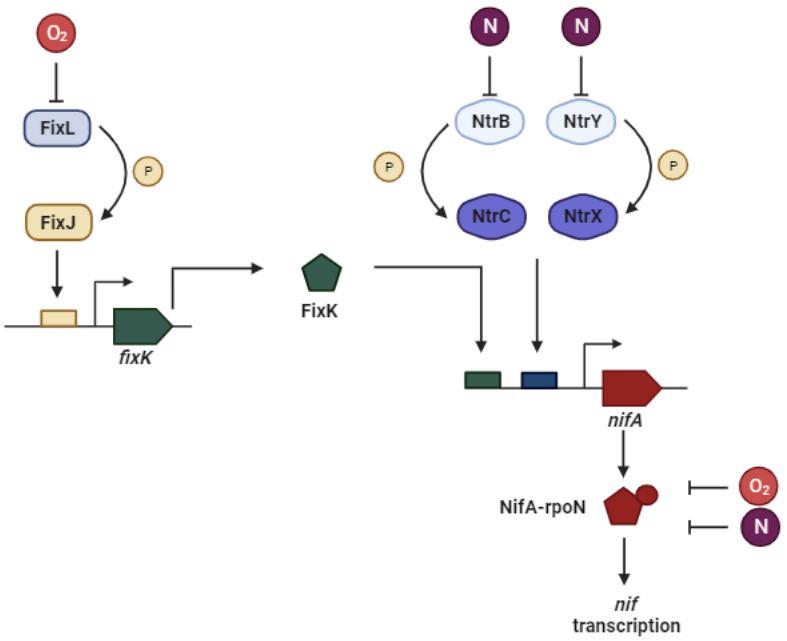


Figure 1.8 Regulatory cascade of free living *nif* transcription in *A. caulinodans*. The oxygen responsive FixL-FixJ two component system controls expression of *nifA* via the FixK transcription factor. In *A. caulinodans nifA* transcription is regulated by the cellular oxygen and nitrogen status. The intracellular nitrogen concentration is sensed by GlnD, which uridylylates PII proteins preventing their repression of the histidine protein kinases NtrB and NtrY, allowing phosphorylation of enhancer binding proteins NtrC and NtrX which induces *nifA* expression and downstream induction of *nif* genes. NifA itself is post-transcriptionally regulated via the cellular oxygen and nitrogen status.

Conditions of nitrogen limitation allow the expression of *nif* genes and nitrogenase activity. These same conditions also upregulate the assimilation of ammonia by the cell, preventing ammonia release to the plant[25,126]. Bacterial glutamine synthetase (GS) assimilates fixed nitrogen by catalysing the condensation of glutamate and ammonia to form glutamine. Fixed nitrogen production and assimilation is energy intensive and is therefore tightly regulated in response to cellular ammonium availability to prevent unnecessary consumption of ATP. The GS enzyme is comprised of 12 identical subunits which are each adenylylated or deadenylylated by a bidirectional adenylyl transferase, encoded by *glnE*, at the Tyr_{397} residue in each subunit[135]. The fully deadenylylated GS form is the most active and becomes slowly inactivated by adenylylation. The post-translational modification state of the PII signal

transduction proteins regulates the activity of the ATase GlnE[129,136]. The PII proteins themselves are regulated by uridylylation/deuridylylation by the bidirectional uridylyltransferase GlnD, which can detect the nitrogen status of the cell via glutamine, a GS product[137]. PII activity is also modulated by ATP/ADP binding that integrates the cellular energy status, and 2-oxoglutarate that reflects sufficient cellular carbon and reflects the cellular C:N ratio. GlnD uridylylates PII proteins under conditions of N-starvation, and alongside ATP and 2-oxoglutarate binding, affects their interaction with cellular targets. The resulting PII-UMP ultimately triggers dephosphorylation of ATase, and hence deadenylylation and activation of GS, alongside upregulation of *nif* genes via the stimulation of NtrC phosphorylation, allowing fixed nitrogen to be assimilated by the cell (*Figure 1.9*)[47,136].

Insertional inactivation of both PII homologues *glnB* and *glnK* produced a mutant unable to activate GS by deadenylylation, thereby repressing N assimilation and driving NH_3-insensitive N_2 fixation and excretion of NH_3 into the growth media[129]. Furthermore, expression of a unidirectional adenylyl transferases *glnE* in ORS571 promoted the inactivation of GS, leading to excretion of fixed NH_3[126]. Deletion of PII genes *glnB* and *glnK* also appeared to derepress the activity of nitrogenase in the presence of high nitrogen media[129]. It has been suggested that in the presence of ammonia, either protein is involved in the repression of nitrogen fixation by post-transcriptional regulation of NifA (*Figure 1.8*)[138]. PII protein regulation of NifA has been supported by introducing mutations identified in *Rhodobacter capsulatus* into the GAF domain of NifA which recovered 50% activity in the presence of ammonium[99,139]. Therefore, the engineering of *Azorhizobium caulinodans* as a diazotrophic inoculant for agriculture, despite its versatility, requires consideration of several regulatory steps to overcome the native regulation for nitrogen fixation and ammonia assimilation.

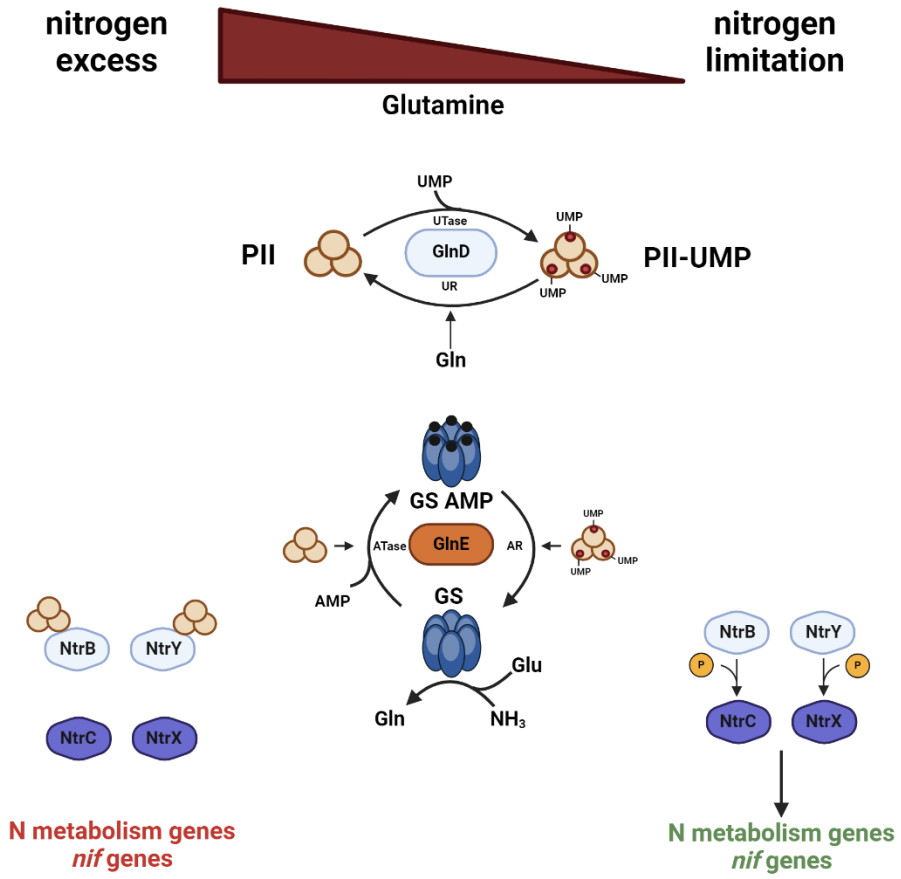


Figure 1.9 Nitrogen regulation in *A. caulinodans*. Regulatory outputs instigated by high nitrogen (left) and nitrogen deficiency (right). Under nitrogen limiting conditions, the intracellular concentration of glutamine is low, which coupled with 2-oxoglutarate binding (red circles), stimulates the Utase GlnD which uridylylates PII proteins GlnB and GlnK. PII-UMP stimulates the adenylyl removal activity of GlnE which controls GS activity. Deadenylylated GS is fully active allowing glutamine synthesis from ammonium and glutamate. The uridylylated PII proteins are unable to bind to histidine protein kinases NtrB and NtrY, preventing their phosphatase activity, consequently leading to the phosphorylation of enhancer-binding proteins NtrC and NtrX for downstream induction of nitrogen metabolism and *nif* genes. Conversely, when the intracellular concentration of glutamine is relatively high, this stimulates the uridylyl-removing activity of GlnD. The resulting deuridylylated PII protein promotes the ATase activity of GlnE leading to GS adenylylation (black dots) preventing nitrogen assimilation. Furthermore, PII interacts with NtrB/Y, stimulating dephosphorylation of NtrC/X, preventing transcription of nitrogen metabolism and fixation genes.

1.2.5 *Enterobacter radicincitans*

A bacterium receiving increasing interest for PGP is *Enterobacter radicincitans*, which was originally isolated from the phyllosphere of winter wheat[140]. However, inoculation of *Triticum aestivum* cultivars with *E. radicincitans* showed it was capable of colonising the inside of the root as well as the inside of leaves and stems[141]. Furthermore, single bacterial cells had been observed to be localized in xylem vessels[142]. Field experiments showed roots were colonised 10–100 times greater than shoots in wheat, with about 10^6 cells g^{-1} root and 10^4 cells g^{-1} shoot. In contrast the colonization of shoots was higher in rye (*Secale cereale*) and barley (*Hordeum vulgare*)[141]. The strain was found to possess nitrogen fixation activity and produce the phytohormones cytokinin and auxin[143]. Furthermore, the strain *E. radicincitans* DSM 16656 was able to induce the priming of defence related pathways in plant cells via salicylic acid or jasmonate/ethylene signalling to improve host protection against potential pathogens[144]. Analysis of 16S rRNA gene sequence and *rpoB* gene sequence identified the bacterium as an *Enterobacter* species, where it was classified as *E. radicincitans*[140]. The PGP characteristics of *E. radicincitans* increase the growth of roots and shoots, as well as increase yields, upon the inoculation of various plant species[141,144,145].

Unlike other PGP *Enterobacter* species isolated, strain DSM16656 is capable of fixing nitrogen. Its genome sequence has a complete *nif* operon, alongside genes for phosphate transport (*pstSCAB*) and siderophore production to harvest iron (*fhuFCDBE*), which will increase the availability of phosphate and iron repectively for PGP[146]. However, nitrogen fixation was still inhibited by fixed ammonia in *E. radicincitans,* as seen in other associative diazotrophs capable of free living N_2 fixation[98,147]. Analysis of the DSM16656 *nif* cluster shows it contains the genes *nifUBALMVSNETKDHJ*[146]. The presence of *nifL* suggests it regulates the transcriptional activator NifA in a similar manner to free living diazotrophs *Klebsiella pneumoniae* and *Azotobacter vinelandii*. Both these species possess a copy of *nifL*, an anti-

activator flavoprotein which interacts with NifA in response to the cellular oxygen and fixed nitrogen status[148]. In these strains and other gamma-proteobacteria, NifL forms an inhibitory complex with NifA which prevents the ATPase activity of the AAA^+ domain of NifA[149]. In both *K. pneumoniae* and *A. vinelandii* the GAF domain of NifA is bound by NifL[86,148]. The NifL repressor contains a flavin-adenine dinucleotide domain which detects the redox status of the cell and regulates the ability of NifL to inhibit NifA activity in response[148]. The interaction between NifL and NifA also integrates nitrogen regulation into *nif* gene expression. In *K. pneumoniae,* expression of *nifLA* and *glnK* is activated by NtrC under nitrogen limitation, via GlnB uridylylation allowing NtrB phosphorylation of NtrC, as in *A caulinodans*[150,151]. Therefore, nitrogen regulation is the first step in the regulatory cascade of *nif* gene activation. Secondly, GlnK itself regulates the interaction of NifL with NifA. Under nitrogen-limiting conditions, when GlnK is uridylylated it binds NifL and prevents inhibition of NifA activity[152].

However, in *A vinelandii,* the converse is true and the non-uridylylated form of GlnK is required for NifL to inhibit NifA, forming a GlnK-NifL-NifA complex under nitrogen excess[149,153]. Furthermore, in *A. vinelandii* the GAF domain of NifA binds 2-oxoglutarate, in the absence of non-uridylylated GlnK. 2-oxoglutarate acts as a proxy for nitrogen limiting conditions, thereby preventing NifA from interacting with NifL and the repression of *nif* gene transcription, integrating cellular carbon and nitrogen regulation of *nif* activity on NifA[154]. The *K. pneumoniae* and *A. vinelandii* regulatory systems demonstrate that the mechanism by which PII signal-transduction proteins regulate NifA activity differs from organism to organism. It is unclear though which method NifA activity is modulated by NifL and PII proteins in regulating *nif* gene expression in *E. radicincitans*. Deletion of *nifL* in the closely related *Kosakonia sacchari* endophyte removed inhibition of NifA and led to ammonia and oxygen insensitive nitrogen fixation, suggesting a similar strategy would function in *E. radicincitans*[155].

1.3 Plant Growth Promotion Phenotypes

Rhizobacteria can improve the growth of associated plant hosts by more mechanisms than just the delivery of fixed nitrogen. As previously discussed, some bacteria have been identified as producing plant growth hormones such as IAA and cytokinin, alongside phosphate solubilisation, and siderophores for iron scavenging[11,156]. Phosphorus often limits plant growth even though it is abundant in soil, as plants require phosphate in a soluble form as either $H_2PO_4^-$ or HPO_4^-, however it is normally bound to metals in the soil[157]. Phosphorus is a major essential macronutrient for plants and is often supplied in synthetic fertilisers alongside nitrogen and potassium[158]. Mycorrhizal fungi associating with plants usually supply plant hosts with solubilised phosphate, and in return plants provide the hyphae with a carbon source[159,160]. However, plant-mycorrhizal associations are severely damaged by fertiliser and pesticide application[161,162]. Rhizobacteria make phosphorus more accessible from the soil by secretion of organic acids, thereby lowering soil pH and solubilising inorganic phosphorus[163]. Gluconic acid is a common organic acid used for mineral phosphate solubilization; it chelates the cations bound to phosphate, thus making the phosphate available to plants[164]. However, this process requires oxidation of glucose to gluconic acid by the bacteria and therefore requires vast amounts of carbon input to be effective at a large scale, so it is unlikely to be used in agricultural inoculants[165]. Organic phosphate corresponds to 20–30% of the total amount of phosphate found in soil. It is mainly derived from biomass, as a constituent biomolecule in animal, plant, and microbial debris[166]. Bacteria can also access organic phosphate soil reservoirs by producing enzymes, such as alkaline and acid phosphatases, phytases, phosphonatases, nucleases and phosphodiesterases[167,168]. Enzymatic release of phosphorus is far less carbon intensive and is therefore a much more favourable strategy to pursue for engineered strains[166].

Phytohormones produced by rhizobacteria can not only influence plant development but can improve host-plant tolerance to abiotic stress, improve nutrient acquisition, provide

protection from pathogenic microbes, and improve the colonisation of rhizobacteria itself[144,169]. The nutrient rich environment of the rhizosphere attracts diverse microbes which synthesise a variety of biologically active compounds including the plant hormones auxin, cytokinin, gibberellin, and ABA[156]. Therefore, microbial production of phytohormones such as auxin (IAA) has been highly researched, even producing commercialised inoculants[119]. Several rhizobacterial species have been investigated for their production of IAA in the rhizosphere. Bacteria produce IAA from tryptophan dependent biosynthesis pathways. IAA is capable of promoting cell elongation, division, and differentiation that when applied to the roots it leads to increased total root surface, enhanced mineral uptake from the soil and root exudation[119,170]. There is increasing evidence that IAA is also used as a signalling molecule in microorganisms as it had been shown to affect microbial gene expression[171]. Microbes producing IAA was originally identified in phytopathogenic bacteria such as *Agrobacterium tumefaciens* and *Pseudomonas savastanoi* which cause plant tumours and galls to provide an advantageous niche to colonise[172,173]. However, IAA production has been identified in both symbiotic nitrogen fixing bacteria, such as *Bradyrhizobium japonicum*[174], and associative diazotrophs such as *Azospirillum brasilense*[117]. In these strains IAA production appears to be induced in the rhizosphere. For example. In *Rhizobium* sp NGR234, flavonoid signal molecules can induce the IAA biosynthesis operon production, alongside nod factor transcriptional regulators NodD1 and NodD2[175]. IAA also appears to impact bacterial gene expression, as IAA was found to induce the auxin biosynthesis gene *ipdC* of *A. brasilense* inducing a positive feedback loop[118]. Auxin accumulation is involved in symbiotic nodule initiation and differentiation, as well as the number of nodules formed[176,177]. As many rhizobia can produce exogenous IAA, it is assumed that they are capable of altering the auxin balance within the roots of plants thereby influencing nodulation[178,179]. A *S. meliloti* IAA-overexpression strain showed increased tolerance to several stresses, and *M. truncatula* plants inoculated with this strain were more

resistant to salt stress and better plant growth under phosphate deficiency[180,181]. The biosynthesis pathway IAA production from *P. savastanoi* has been expressed in *Cupriavidus pinatubonensis*[182]. When inoculated onto *Arabidopsis*, the resulting strain increased lateral root number, root length, fresh weight, and rosette area[182]. Furthermore, it has been shown that auxins can induce the formation of nodule like structures on non-legume land plants in the absence of rhizobial partners[183,184]. IAA and synthetic auxin, 2,4-Dichlorophenoxyacetic acid, were applied to roots and induced nodule-like structures in wheat, rice, and *M. truncatula*[184]. These structures, resemble nodules but can be distinguished by their diffuse meristem, and high cell differentiation in the root cortical cells and vascular bundle. The induced nodule like structures can be colonized via crack-entry of the tumorous structures by endophytic nitrogen-fixing bacteria like *A. caulinodans* and *A. brasilense*[183,185]. Therefore, auxin production by a potential bioinoculant will be valuable for engineering strategies for nitrogen fixation in cereals.

Plant rhizobacteria also contribute to biocontrol via the inhibition of a wide range of phytopathogens, which could be leveraged to reduce the dependency of agriculture on pesticides[186,187]. Pesticides can persist in the environment where they may accumulate to toxic levels, affecting humans and other organisms which consume plant derived organic matter, as well as disrupting existing beneficial plant-microbe interactions[161,188]. At the most basic levels, rhizobia chelate metals such as iron by secreting siderophores alongside the consumption of rhizosphere carbon sources which outcompetes other microbes present and prevents the spread of plant pathogens[186,189]. However, they have also been shown to directly control pathogens in the rhizosphere via the production of antimicrobial compounds, such as phenazines, and volatiles like HCN[156,187,190]. Some strains of *R. leguminosarum* contain the symbiotic plasmid pRL1J1, which carries *nod* and *nif* genes alongside genes for bacteriocin production[190,191]. *Pseudomonas* species have been identified as being able to control competitive pathogens in

the rhizosphere via the production of anti-fungal molecules[192]. Biosynthesis of genes for pyrrolnitrin, a broad-spectrum antifungal molecule, produced by *P. fluorescens* has been shown to antagonise *Rhizoctonia solani* induced damping-off disease in inoculated cotton[193]. Rhizobia have also been reported to produce mycolytic enzymes, such as chitinase, that can hydrolase chitin chains which are a major constituent of fungal cell walls[194,195]. *Rhizobium* species have been isolated which show good chitinase activity against *F. oxysporum* and *Aspergillus* species[195]. Furthermore, chitinase genes from *Serratia marcescens* were transferred to *S. meliloti* enabling it to prevent *R. solani* colonisation of alfalfa[194]. Colonisation of plants by rhizobacteria has also been shown to induce systemic resistance and enhance expression of plant defence-related genes in the plants they colonise, which effectively primes the plant-host against pathogens[196,197]. Induced systemic resistance is similar to innate immunity in humans and is dependent on jasmonic acid and ethylene signalling in the plant, which can be influenced by symbiotic rhizobia[11,197]. However, more research is required to fully elucidate the role symbiotic bacteria play in plant defence response.

1.3.1 Engineering of Bacteria for PGP

Engineering PGP into microbial inoculants has primarily focused on nitrogen fixation due to the crucial role nitrogen plays in plant growth. Several strategies could be employed to engineer the delivery of fixed N to target crops: genes encoding the nitrogenase enzyme complex could be directly engineered into plant cells, or the capacity for nodule formation and symbiosis with efficient N_2 fixing microbes could be transferred to the roots of target cereals[198-200]. Alternatively, control of nitrogen fixation and ammonium excretion could be engineered into symbiotic rhizobia or bacteria which already closely associate with cereals[96,99,201]. The first method used to engineer a diazotrophic strain was the transfer of the *nif* gene cluster from *Klebsiella oxytoca* to *E. coli* in 1972[202]. Since then, the technology and knowledge has advanced to allow more complex genetic engineering feats. Studies have now tested 12 *nif* gene

clusters in 15 bacterial species[99]. However, the productivity of transferred clusters in their recipient strain is still largely affected by the genotype and native regulation of the recipient[99]. The variability in *nif* genes content across diazotrophs is clearly determined by the environmental lifestyle of each bacterium, therefore the minimal *nif* gene required to achieve nitrogen fixation in a donor strain must take this into account. Transfer of minimal gene clusters between diverse bacterial species has been possible, such as the expression of 9 *nif* genes, *nifB*, *nifH*, *nifD*, *nifK*, *nifE*, *nifN*, *nifX*, *hesA* and *nifV,* from *Paneibacillus polymyxa* WLY78 in *E. coli* that resulted in 10% activity of nitrogen fixation relative to the WT *P. polymyxa* strain.

Bacteria which are capable of free living nitrogen fixation tend to express *nif* accessory genes that can assist in electron delivery to the nitrogenase complex and oxygen protection mechanisms. Thereby, the activity of *P. polymyxa nif* genes in *E. coli* was improved to 50% of WT activity by the co-expression of electron transport genes *pfoABfldA*, alongside the nitrogenase Fe-S cluster assembly genes *nifSU* from *K. oxytoca*[203]. Furthermore, strategies to optimise *nif* cluster expression and protein stoichiometry, alongside the removal of native regulation, have been used to improve nitrogenase activity in donors. The *nif* cluster from the free living diazotroph *K. oxytoca* was rebuilt using synthetic well characterised genetic parts to maintain control of N_2 fixation whilst eliminating internal regulation. Refactoring also involved the removal of non-essential genes and regulatory proteins such as NifLA from the cluster, followed by divergent refactoring of the WT codon sequence. The resulting genes were organised into artificial operons and placed under control of an orthogonal T7 polymerase alongside synthetic RBS and terminator sequences. The resulting synthetic *nif* cluster achieved 10% of the nitrogenase activity relative to the WT but exhibited minimal expression tolerance between parts. However, the synthetic cluster maintained low levels of activity in the presence of ammonia, demonstrating the feasibility of synthetic clusters to overcome regulation[204].

The activity of a synthetic refactored cluster was improved by combinatorial design, as refactoring *nif* operons allows the large-scale iterative process of testing and swapping of genetic parts. The combinatorial design process was applied to the *K. oxytoca nif* cluster to produce variant v2.1 which achieved 57% of WT rates of N_2 fixation in *K. oxytoca* Δ*nif.* However, when transferred to *E. coli,* the optimised cluster resulted in 7% WT activity, highlighting the differences in genetic part functionality between hosts[205]. The complexity of diazotroph *nif* gene clusters and the necessity to maintain the stoichiometry of protein expression in operons presents a complex challenge when overhauling the design of *nif* clusters. A second strategy utilising native translation initiation signals and preserving native *nif* operon structure demonstrated WT levels of nitrogen fixation in *E. coli*. The *K. pneumoniae nif* gene cluster was re-assembled as native operon based BioBrick parts, resulting in 100% of WT expression in *E. coli.* To exert heterologous control of *nif* expression and remove regulation, the native σ^{54}-factor promoters were replaced with T7-dependent promoters with similar expression profiles, resulting in 42% nitrogenase activity relative to the native σ^{54} dependent *nif* operons tested in *E. coli*[206]. Furthermore, the T7 controlled *nif* cluster bypassed native fixed nitrogen and oxygen regulatory circuits but was still subjected to physiological limitations, such as the oxygen sensitivity of nitrogenase and post-transcriptional regulation in response to fixed nitrogen[206].

Despite the ability of *E. coli* carrying *nif* genes to fix nitrogen, it is not an agriculturally relevant host, therefore for delivery of fixed nitrogen to cereals the engineering of *nif* genes in symbiotic or endophytic species is much more relevant. The refactored *K. oxytoca nif* cluster v2.1 was put into the cereal endophytes *P. protogens* Pf-5 and *Rhizobium sp*. IRBG74. RNA-sequence profiling of the clusters showed that the genetic parts were less active in these strains resulting in poor correlation with the *nif* gene expression rates in native *K. oxytoca*[99]. A second *nif* cluster v3.2 with native operon structure to preserve expression ratios and rhizobial

optimised genetic components was tested, resulting in low rates of N_2 fixation in these endophytic strains[99]. Transfer of native *nif* clusters between endophytic hosts has also been tested, however these result in constitutive activity or maintain strong repression in the presence of fixed nitrogen, resulting in low activity and difficulty in controlling *nif* gene activity[99]. Typically, transfer of *nif* clusters between more distantly related bacteria, such as gamma and alpha-proteobacteria, results in the generation of non-fixing strains due to transcriptional incompatibility. Transfer of the *P. stutzeri nif* cluster to *P. protegens* Pf-5, a cereal biocontrol inoculant which lacks *nif* genes, results in nitrogen insensitive fixation and ammonium release to plant hosts under nitrogen-starvation conditions[207,208]. However, the *P. stutzeri nif* cluster exhibited constitutive activity in Pf-5, imparting a severe fitness cost on the bacterium rendering it uncompetitive in the rhizosphere[208].

Another possibility for regulating nitrogen fixation in diazotrophs for agronomic benefit is the use of controllers for native *nif* expression. The convergence of regulatory signals on the master transcriptional regulator NifA, in association with the RNA-polymerase sigma factor RpoN, provides an excellent opportunity to regulate *nif* clusters in bacterial inoculants[130]. However, aside from transcriptional regulation by NtrC, many diazotrophs possess regulation at post-transcriptional levels which must be addressed to control N_2 fixation[83]. The N-terminal domain of *A. brasilense* NifA acts as a negative regulator of the NifA activity in response to fixed nitrogen, whilst the uridylylated PII protein is required to activate NifA under nitrogen limitations[209]. It was demonstrated that a mutation in Tyr^{18} residue in this NifA domain led to an active protein which was insensitive to nitrogen conditions[210]. A similar result was achieved in *Herbaspirillum seropedicae* using a truncated NifA protein lacking the regulatory domain[211]. Regulation of NifA activity in *Rhodobacter capsulatus* is repressed by ammonium at the post-translational level independent of NtrC. Analysis of a mutant carrying amino acid substitutions in the N-terminal domain of NifA showed ammonium tolerant activation of *nif* genes[139].

Mutation of the NifA GAF domain was extended to develop an ammonium insensitive NifA controller in *A. caulinodans.* Induction of a controller co-expressing *nifA* and *rpoN* lead to complete recovery fixation in a Δ*nifA* strain but exhibited only 5% activity in the presence of ammonium. Therefore, the mutations identified in *R. capsulatus* NifA were identified and mutated in the *A. caulinodans* NifA controller which recovered 50% of N_2 fixation under high nitrogen conditions[99]. Use of a nitrogen insensitive transcriptional regulator offers a promising strategy of engineering control of nitrogen fixation in diazotrophic inoculants.

Aside from engineering nitrogen fixation, PGP by a diazotrophic strain is only possible if fixed nitrogen is delivered to the host. In symbiotic nodule fixation, ammonia is transferred from bacteroids via simple diffusion or through protein channels in the symbiotic membrane where it is incorporated by the host[32]**.** However, all known associative diazotrophic strains display tight regulation of nitrogen fixation and assimilation and do not deliver excess to plant hosts, which must be overcome in engineered inoculants to benefit crop growth. Engineering diazotrophs which release ammonia have been demonstrated in free living N_2 fixing strains such as *A. vinelandii* which possess NifL-NifA regulatory systems[148]. NifL is an anti-activator which integrates cellular oxygen, carbon, and nitrogen status signals to regulated activity of NifA and *nif* expression and is bound by GlnK signal transduction protein in response to the cellular nitrogen status[152,212]. Deletion of *nifL* results in constitutive *nif* gene expression, due to removal of regulatory inputs on NifA activity, and ammonia secretion albeit at a substantial fitness burden to the bacterium[153,213,214]. As shown in *A. caulinodans,* mutagenesis was performed on *A. vinelandii nifA* to identify NifA variants which were able to escape fixed nitrogen regulation by NifL without the cost of constitutive *nif* expression[86,215]. A NifA-E356K substitution was shown to enable expression of nitrogenase in the presence of excess fixed nitrogen but was dependent on the cellular carbon status for release of ammonia[214]. Further strategies for engineering ammonia excretion have focused on GS biosynthetic activity and its

regulatory cascade. In *A. caulinodans*, insertional inactivation of both PII homologues *glnB* and *glnK* produced a mutant that was unable to activate GS by deadenylylation, thereby driving NH_3-insensitive N_2 fixation and excretion of fixed nitrogen[129]. However, this strategy is not effective in all diazotrophs as PII regulates NifA and nitrogenase activity and is essential for growth in some strains[126,129,136]. A successful strategy for engineering nitrogen fixation has focused on inhibition of GS activity. Bacterial GS is regulated by adenylylation or deadenylation by a bidirectional adenylyl transferase, encoded by *glnE*. The directionality of the adenylyl transferase is regulated by GlnD-PII cascade in response to cellular nitrogen status (*Figure 1.9*). To engineer GS inactivity, unidirectional adeyltransferases that encoded only the C-terminal adenylylation domain and are only capable of adenylylation of GS were expressed in *Azospirillum brasilense ΔglnE* and resulted in excretion of ammonia[216,217]. Modulation of GS activity has been demonstrated in other diazotrophs due to the conserved function of GlnE, and coupled with nitrogen insensitive NifA in *A. caulinodans,* also produced an ammonia excretion phenotype[126]. It also appears that inactivation of GS, and therefore glutamine biosynthesis, removes fixed nitrogen regulation of N_2 fixation in diverse diazotrophic species, possibly by disrupting glutamine dependent posttranscriptional feedback of NifA and nitrogenase[126,138]. Utilising strategies for both control of nitrogen fixation and ammonium secretion will be key for effective PGP in engineered inoculants.

1.4 Plant Host-Specific Control of Gene Expression

Expression of *nif* genes in an engineered diazotrophic strain must be tightly controlled due to the energetic demands of nitrogen fixation, rendering constitutive expressing strains as suboptimal colonisers of the rhizosphere and selecting for silencing mutations[155,214]. Tuneable expression systems have been used for a variety of applications in microbiology and are essential to regulate orthogonal gene expression to preserve the fitness of host bacterium[218-220].

To ensure expression of N_2 fixation genes and delivery of ammonia only in the rhizosphere of target crop species, a host specific signal is required to regulate gene expression in engineered bacterial strains. Agriculturally relevant signals which regulate characterised transcription factors and their cognate promoters have already been characterised and used to control *nif* gene expression[99]. To control *nif* genes in the proximity of plants, root exudate signals such as the plant hormone salicylic acid, the naringenin flavonoid, and vanillic acid antimicrobial were used to make inducible controller plasmids in *P. protegens* Pf-5, *R.sp* IRBG74, and *A. caulinodans*. Furthermore, to study if genetically engineered plants could alter their microbiome, *Arabidopsis thaliana* secreting opines, a carbon signal induced in tumours by pathogenic *Agrobacterium*, was found to enrich the rhizosphere of cognate bacterium that could use the signal as a carbon and nitrogen source to aid in colonisation[221,222]. However, a limitation of plant derived signalling is the ability of diverse non-target rhizosphere bacterium to benefit from non-exclusive plant exudates[223,224]. Furthermore, the utilisation of signals from phytopathogenic bacteria is undesirable in candidate crop species. To overcome this, the rhizobia specific inositol rhizopine compounds were investigated as an orthogonal trans-kingdom signal for controlling bacterial gene expression in the rhizosphere.

Rhizopines *scyllo*-inosamine (SI) and 3-*O*-methyl-*scyllo*-inosamine (3-*O*-MSI) are synthesised by bacteroids in legume symbiosis with *R. meliloti* and *R. leguminosarum* species. In these species the genes for rhizopine synthesis and catabolism are located on the Sym plasmids[225]. Analysis of the *mos* biosynthesis operon demonstrates that 3-*O*-MSI is synthesised by a two-step process involving an ononitol dehydrogenase encoded by *mosDEF* and an amino-transferase encoded by *mosB* which are expressed from NifA specific promoters (*Figure 1.10*)[226,227]. Therefore, rhizopines produced in nodules can be catabolised by free living rhizobial strains carrying the catabolism operon *mocRABCDEF*, enhancing the competitive ability of cognate strains within the rhizosphere[228]. Rhizopine catabolism has been confirmed

by competition studies whereby strains possessing the ability to produce and catabolise rhizopines occupy a higher percentage of root nodules[229]. Rhizopines are ideal as a plant derived signal for control of bacteria because the biosynthesis and catabolism genes are well characterised, it has shown to be exuded into the rhizosphere, and it is rare in bacterial species[230]. To use the rhizopine signal in transgenic plants, *Medicago* and barley lines expressing a synthetic biosynthesis pathway for SI have been developed[227]. To construct a synthetic pathway for SI production in plants, inositol dehydrogenase (IdhA) from *R. leguminosarum* and *scyllo*-inosose:L-glutamate aminotransferase (MosB) were transferred to plants under control of constitutive plant promoters CaMV 35s and *Lotus japonicus* Ubiquitin1, respectively (*Figure 1.9*). *M. truncatula* producing SI from the synthetic pathway were able to induce *R. leguminosarum* Rlv3841 carrying a rhizopine Lux biosensor under control of the MocR regulator[227,231]. Rhizopine production was also confirmed in engineered barley plants, whereby *idhA* was expressed under the control of *Zea mays* ubiquitin1 promoter and *mosB* by *Oryza sativa* ubiquitin1 promoter, demonstrating the ubiquity of the signal for plant control of bacterial gene expression[227].

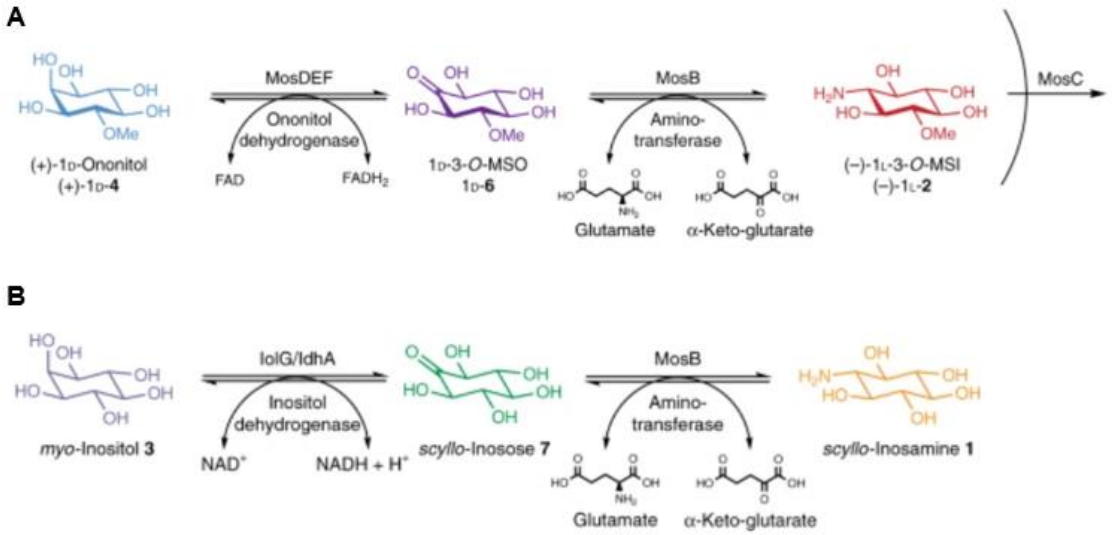


Figure 1.10 A The natural pathway of 3-*O*-MSI biosynthesis from rhizopine producing rhizobium *S. meliloti* L5-30. **B** The synthetic pathway for rhizopine production engineered into transgenic lines, utilising bacterial inositol dehydrogenase to produce the intermediate *scyllo*-inosose which is transaminated by the MosB aminotransferase from *S. meliloti* to *scyllo*-inosamine. Adapted from Geddes *et al*., 2019[227].

Exudation of SI from the roots of transgenic plants can be sensed by bacteria carrying a receiver plasmid designed to mimic the rhizopine induction of catabolism genes in *S. meliloti* L5-30. In this strain, rhizopine is transported into the bacterium across the cytoplasmic membrane via the solute binding protein MocB interacting with the transmembrane domain of the ATP-binding cassette (ABC) transporter encoded by *intBC* genes from *R. legumonisarum* Rlv3841 (*Figure 1.11*). In rhizopine catabolising bacteria, the transcription factor MocR regulates the expression of *mocBA* from the upstream P*mocB* promoter in the presence of rhizopine. Therefore, in the bacterial SI plasmid sensor, cytoplasmic rhizopine binds MocR which can regulate expression of genes placed downstream from the P*mocB* promoter (*Figure 1.11*). A rhizopine biosensor plasmid can therefore be used for specific plant-dependent regulation of *nif* and other PGP genes in engineered bacterium in the rhizosphere of target crop species. Placing the *nifA* transcriptional regulator under control of P*mocB* in an associative

diazotrophic strain allows plant dependent control of nitrogen fixation in the rhizosphere of *RhiP* cereals and would prevent induction and growth promotion of nontarget species[201].

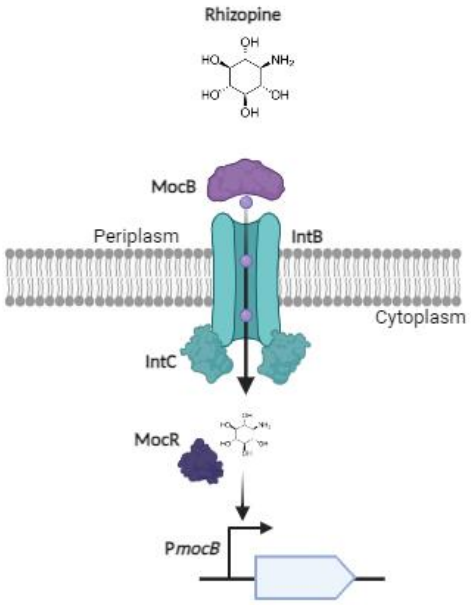


Figure 1.11 Bacterial sensing of rhizopine SI by *S. meliloti* L5-30 solute-binding protein MocB interacting with transmembrane components of the *Rlv*3841 ABC inositol transporter IntBC for the transport of rhizopine into the cytoplasm where it is bound by the MocR transcriptional regulator for expression of genes from P*mocB*.

1.5 Objectives of This Work

Signalling in the rhizosphere between legumes and their cognate symbionts is of paramount importance for subsequent symbiosis formation and initiation of nitrogen fixation mechanisms. The aim of this work is to demonstrate the use of an engineered signal to control nitrogen fixation in rhizobacteria for plant growth promotion in cereals. Therefore, the synthetic rhizopine signal was tested for induction and control of gene expression and nitrogen fixation in diverse diazotrophic species. The objective of Chapter 3 was to demonstrate control of nitrogen fixation in the associative diazotroph *A. caulinodans in vitro*. Rhizopine receiver

plasmids were tested for their induction in this strain in response to the synthetic signal. Induction by rhizopine was followed by demonstration of the engineered signalling circuit's ability to drive free living nitrogen fixation by placing the *nifA* transcription factor under control of a rhizopine responsive promoter. Development of an efficient and competitive diazotrophic strain for use in agriculture will likely involve the establishment of nodule forming cereals. Therefore, rhizopine control of gene expression was explored in Chapter 4 in the symbiotic rhizobium, *S. meliloti,* for control of nitrogen fixation in the nodule environment.

The transfer of gene clusters between species is an exciting prospect in synthetic biology and a strategy that can be employed to engineer strains that can fix nitrogen under free living conditions when they do not so naturally. *Nif* genes are under stringent regulatory control due to the cellular metabolic cost associated for N_2 fixation, thus transfer of refactored gene clusters between species can eliminate this repression and have been demonstrated for refactored *K. oxytoca nif* clusters in *Rhizobium sp* IRBG74. Therefore, we tested this strategy in *S. meliloti* using previously characterised engineered clusters to see if this method could be used to develop control of symbiotic nitrogen fixation in this strain.

Rhizobacteria provide PGP mechanisms other than supplying fixed nitrogen to the host plant. These PGP phenotypes could be engineered into an effective nitrogen fixing rhizobium strain and controlled alongside *nif* genes, thereby producing a more effective bacterial inoculant for agriculture. Furthermore, rhizopine signalling has some drawbacks as it is limited to alpha-proteobacteria. Therefore, work in Chapter 5 tested engineered auxin biosynthesis from bacterial strains and characterises operons for secondary metabolite biosynthesis which were used as relay signals to amplify the plant-host derived rhizopine signal to more diverse rhizobacteria for PGP of cereals.

Chapter 2

Materials and Methods

Table 2.1 Bacterial Strains Used in This Study

Species	Name	Description	Chapter	Reference
E. coli	DH5 alpha	Competent E. coli strain; F-deoR endA1 recA1 relA1 gyrA96 hsdR17(rk-mk+) supE44 thi-1 – phoA Δ(lacZYAargF) U169 Φ80lacZΔM1	3-5	Bioline
E. coli	ECD1000 pir-116	Strain for maintenance of R6K plasmids	3-5	Lucigen
S. meliloti	CL150	Sm1021 derivative corrected for nonfunctional *ecfR1* and *pstC* genes, *Strep*^{r}	4-5	[232]
S. meliloti	2011	streptomycin-resistant derivatives of the wild-type *S. meliloti* isolate SU47, *Strep*^{r}	5	[233]
S. meliloti	1021	streptomycin-resistant derivatives of the wild-type S. meliloti isolate SU47, *Strep*^{r}	5	[234]
P. fluorescens	SBW25	Pseudomonad isolated from the phytosphere of sugar beet	5	[235]
E. ludwigii	AA4	*Enterobacter ludwigii* AA4, isolated from the root of maize	5	[236]
Streptomyces sp	A34	*Streptomyces* soil isolate	5	[237]
A. brasilense	FP2	Root associated diazotroph *Azospirillum brasilense* WT, *Amp*^{r}	5	[238]
R. leguminosarum	3841	Rhizobium leguminosarum bv. Viciae; *Strep*^{r} derivative of strain 300	5	[239]
Rhizobium sp.	IRBG74	*Rhizobium* sp. Isolated from root nodules of the aquatic legume Sesbania cannabina	4,5	[240]
M. loti	R7A	*Lotus* nodulating strain	5	[241]
R leguminosarum	*trifolii*	symbiotic rhizobium that nodulates *Trifolium* sp plants	5	[242]

A. vinelandii		Model organism for free living nitrogen fixation	5	[243]
B. japonicum		Soybean symbiotic rhizobium, *Cm*^{r}	5	[244]
M. ciceri	WSM1497	3-oxo-C6-HSL producing strain	5	[245]
E. radicincitans	DSM16656	PGP bacterium isolated from the phyllosphere of winter wheat, *Nit*^{r}	5	[140]
A. caulinodans	ORS571	WT *Azorhizobium caulinodans, Amp*^{r}	3,5	[120]
A. caulinodans	Δ*nifA*	ORS571 *nifA* deletion strain, *Amp*^{r}	3,5	[99]
A. caulinodans	OPS1204	ORS571 carrying SI biosensor pOPS0889	3	[201]
A. caulinodans	OPS1079	ORS571 carrying SI biosensor pOPS0761	3	[201]
A. caulinodans	OPS2202	ORS571 carrying SI biosensor pOPS1495	3	[201]
A. caulinodans	OPS1077	ORS571 carrying SI biosensor pOPS0759	3	[201]
A. caulinodans	OPS1626	ORS571 carrying SI inducible *nifA* plasmid pOPS1122	3	[201]
A. caulinodans	OPS2624	ORS571 carrying SI inducible *nifA* $_{L94Q/D95Q}$ plasmid pOPS1476	3	This work
A. caulinodans	OPS2622	ORS571 carrying SI inducible *nifA* plasmid pOPS1122 with P*nifH::mCherry* reporter pOPS1218	3	This work
A. caulinodans	OPS2620	ORS571 carrying SI inducible *nifA* $_{L94Q/D95Q}$ plasmid pOPS1476 with P*nifH::mCherry* reporter pOPS1218	3	This work
A. caulinodans	OPS2222	ORS571 ΔglnB mutant	3	[126]
A. caulinodans	OPS2379	ORS571 ΔglnK mutant	3	[126]
S. meliloti	OPS2632	pOPS1536 naringenin-inducible *gfp*	4	This work
S. meliloti	OPS2631	Taurine inducible *gfp* pLMB509	4	This work
S. meliloti	OPS2630	2,4-DAPG inducible *gfp* pOPS1537	4	This work
S. meliloti	OPS2627	pOPS1577 aTc inducible *gfp*	4	This work
S. meliloti	OPS2629	pOPS1631 IPTG inducible *gfp*	4	This work

S. meliloti	OPS2634	pZH19 T7 RNAP controller with pOPS1716 *sfGFP* reporter	4	This work
S. meliloti	OPS1507	CL150 Δ*nifA*	4	Timothy Haskett
S. meliloti	OPS3106	T7 controller with V2.1 refactored cluster	4	This work
S. meliloti	OPS3107	T7 controller with V3.2 refactored cluster	4	This work
P protegens Pf-5	OPS3537	T7 controller with V2.1 refactored cluster	4	This work
P protegens Pf-5	OPS3538	T7 controller with V3.2 refactored cluster	4	This work
Rhizobium sp. IRBG74 Δ*nif*Δ*hsdR*	OPS3507	T7 controller with V2.1 refactored cluster	4	This work
Rhizobium sp. IRBG74 Δ*nif*Δ*hsdR*	OPS3508	T7 controller with V3.2 refactored cluster	4	This work
S. meliloti	OPS2633	pBBR1 SI biosensor pOPS0889	4	This work
S. meliloti	OPS1726	RK2 SI biosensor pOPS1052	4	This work
S. meliloti	OPS1835	WT CL150 with mTn7 *attB* integration site	4	This work
S. meliloti	OPS3099	Integrated SI biosensor	4	This work
S. meliloti	OPS3307	SI biosensor pOPS1998	4	This work
S. meliloti	OPS2879	CL150 LP with integrated P*J23104::mCherry*	4	This work
S. meliloti	OPS3289	pOPS0889 constitutive mCherry	4	This work
S. meliloti	OPS3509	pOPS1052 constitutive mCherry	4	This work
S. meliloti	OPS3503	pOPS1998 constitutive mCherry	4	This work
S. meliloti	OPS3101	integrated SI biosensor was complemented with pOPS0759 carrying *mocR*-P*mocB::gfp*	4	This work
S. meliloti	OPS3065	OPS3101 with pOPS1879 P*J23104::mCherry* plasmid	4	This work
S. meliloti	OPS2877	pOPS1791 for SI inducible *nifA* expression	4	This work
S. meliloti	OPS3509	CL150 P*J23104::mCherry* marked strain carrying the RK2 SI biosensor pOP1052	4	This work

S. meliloti	OPS3259	CL150 with pOPS1987 *PmocB::mCherry* plasmid	4	This work
S. meliloti	OPS3103	RK2 constitutive IAA plasmid pOPS0667	5	This work
S. meliloti	OPS3102	pBBR1 constitutive IAA plasmid pOPS0666	5	This work
P fluorescens SBW25	OPS3504	pRK2 constitutive IAA plasmid pOPS0667	5	This work
P fluorescens SBW25	OPS3505	pBBR1 constitutive IAA plasmid pOPS0666	5	This work
S. meliloti	OPS1864	*S. meliloti* with integrated P*J23115::mocBintBC-mocR*-P*mocB::gfp*	5	This work
S. meliloti	OPS3104	Constitutive 2,4-DAPG biosynthesis plasmid pOPS0910	5	This work
S. meliloti	OPS3105	CL150 with tn7 integrated SI biosensor carrying SI inducible 2,4-DAPG plasmid pOPS0909	5	This work
S. meliloti	OPS3511	RK2 SI inducible 2,4-DAPG plasmid pOPS2093	5	This work
S. meliloti	OPS3513	pBBR1 SI inducible 2,4-DAPG plasmid pOPS2095	5	This work
S. meliloti	OPS3512	RK2 SI inducible *phlACB* with P*J23106::phlD-phlE*	5	This work
S. meliloti	OPS3514	pBBR1 SI inducible *phlACB* with P*J23106::phlD-phlE*	5	This work
S. meliloti	OPS3515	pOPS2097 RK2 SI inducible WSM1497 *traR-traI*	5	This work
S. meliloti	OPS3517	pOPS2099 SI inducible RBS_{28}-*traR-traI*	5	This work
S. meliloti	OPS3518	pOPS2100 SI inducible P*mocB::traI* AHL plasmid	5	This work
E. radicincitans	OPS3689	pOPS2155 *luxR-Plux::gfp*	5	This work
A. caulinodans	OPS3691	pOPS2155 *luxR-Plux::gfp*	5	This work
E. radicincitans	OPS3472	*ΔnifL*	5	This work
E. radicincitans	OPS3473	*ΔnifA*	5	This work

E. radicincitans	OPS3474	*ΔnifLA*	5	This work
E. radicincitans	OPS3681	2,4-DAPG inducible *E. radicincitans nifA* controller in *ΔnifLA* strain	5	This work
E. radicincitans	OPS3682	AHL inducible *E. radicincitans nifA* controller in *ΔnifLA* strain	5	This work
E. radicincitans	OPS2510	*E. radicincitans* with integrated P*J23104::mCherry*	5	Carolin Schulte
E. radicincitans	OPS3685	Constitutive mCherry marked strain carrying AHL inducible *gfp* plasmid pOPS2155	5	This work
E. radicincitans	OPS3692	Constitutive mCherry marked strain carrying 2,4-DAPG inducible *gfp* plasmid pOPS1537	5	This work

Table 2.2 Bacterial Plasmids Used in This Study

Name	Description	Resistance	Chapter	Reference
pRK2013	Helper plasmid for mobilization of non-self-transmissible plasmids	Kan/Neo	3-5	Addgene
pTNS3	Transposase delivery plasmid for integration of mini-Tn7 cassettes	Amp	3-5	Addgene
pOPS0761	P*mocB* driving *gfp* expression	Kan/Neo	3	[201]
pOPS1495	P*J23115::mocB*-P*mocB::gfp*	Kan/Neo	3	[201]
pOPS0601	Domesticated *R. leguminosarum intBC* genes	Kan/Neo	3	[201]
pOPS0889	P*J23115*::*mocBintBC-mocR-PmocB::gfp*	Kan/Neo	3	[201]
pOPS0759	*mocR-PmocB::gfp*	Kan/Neo	3	[201]
pOPS1122	pOPS0889 with *PmocB::nifA-rpoN-gfp*	Kan/Neo	3	This work
pOGG290	Domesticated *A. caulinodans nifA*	Kan/Neo	3	[201]
pOPS1476	pOPS0889 with *PmocB::nifA* $nifA_{L94Q/D95Q}$ *-rpoN-gfp*	Kan/Neo	3	This work
pOPS1214	pOGG024 carrying P*nifH1::mCherry*-Tpharma	Tet	3	[201]
pOPS1218	pRK415 carrying P*nifH1::mCherry*-Tpharma	Tet	3	[201]
pOPS1691	*Ac glnB* deletion vector in pK19mobSacB plasmid	Kan/Neo	3	[126]

pOPS1564	*Ac glnK* deletion vector in pK19mobSacB plasmid	Kan/Neo	3	[126]
pOPS1536	naringenin-inducible *gfp*	Tet	4	Timothy Haskett
pLMB509	taurine inducible *gfp*	Gent	4	[219]
pOPS1537	2,4-DAPG inducible *gfp*	Tet	4	Timothy Haskett
pOPS1577	aTc inducible *gfp*	Kan/Neo	4	Timothy Haskett
pOPS1631	IPTG inducible *gfp*	Kan/Neo	4	Timothy Haskett
pZH19	IPTG inducible T7 RNAP	Tet	4	[99]
pOGG024	pBBR1 pL1V-ELT3 backbone	Gent	4,5	[246]
pOGG152	pL0M-T7 promoter	Amp	4	Kyle Grant
pOGG143	pL0M-$RBS_{standard}$	Amp	4	Kyle Grant
pOGG037	pL0M-*sfGFP*	Amp	4	[246]
pOGG039	pL0M-T7 terminator	Amp	4	[246]
pOPS1716	*sfGFP* reporter for T7 RNAP	Gent	4	This work
v2.1	refactored *Klebsiella oxytoca* nitrogen fixation clusters	Kan/Neo	4	[99]
v3.2	refactored *Klebsiella oxytoca* nitrogen fixation clusters	Kan/Neo	4	[99]
pOPS1052	RK2 replicon P*J23115*::*mocBintBC-mocR-PmocB::gfp*	Tet	4	[201]
pOGG093	pL1V-RK2-par-ELT4 vector backbone	Tet	4	[247]
pOPS1744	R6K mini-Tn7 vector	Gent	4	Timothy Haskett
pOPS1740	mini-Tn7 vector for SI biosensor integration with P*mocB::gfp*	Gent	4	[248]
pOPS1998	P*rpoD::intBC* SI biosensor	Kan/Neo	4	[248]
pOPS1531	Mini-Tn7 pUC18R6K delivery vector carrying constitutive P*J23104*::*mCherry* cassette	Gent	4	Beatriz Jorrin
pOPS1879	RK2 plasmid P*J23104::mCherry*	Tet	4	Pilar Velasco
pOPS1791	RK2 SI plasmid with *PmocB::nifA* from *S. meliloti*	Tet	4	This work
pOPS1889	RK2 SI biosensor plasmid lacking *gfp*	Tet	4	[248]
pOPS1987	RK2 SI plasmid with *PmocB::mCherry*	Tet	4	This work
pOGG192	Domesticated *iaaM*	Spec	5	Barney Geddes
pOGG193	Domesticated *iaaH*	Spec	5	Barney Geddes
pOGG021	pLV1-F1 golden gate vector	Amp	5	[246]
pOGG121	pL0M-P-*J23106*	Spec	5	Kyle Grant

pOGG144	pL0M-RBS1	Spec	5	Kyle Grant
pOGG160	pL0M-F2S terminator	Spec	5	Kyle Grant
pOGG059	pLV1-R2 golden gate vector	Amp	5	[246]
pOGG122	pL0M-P*J23115*	Spec	5	Kyle Grant
pOGG143	pL0M-RBSstandard	Spec	5	Kyle Grant
pOGG156	pL0M-DT15 terminator	Spec	5	Kyle Grant
pOGG234	pL1M-*iaaM* expression cassette	Amp	5	Barney Geddes
pOGG235	pL1M-*iaaH* expression cassette	Amp	5	Barney Geddes
pOGG096	pLV2-RK2-par medium copy vector	Kan/Neo	5	[247]
pOPS0667	RK2 constitutive IAA biosynthesis plasmid	Kan/Neo	5	Barney Geddes
pOGG027	pLV2-BBR1 high copy vector	Kan/Neo	5	[247]
pOPS0666	pBBR1 constitutive IAA biosynthesis plasmid	Kan/Neo	5	Barney Geddes
pOGG072	pL0M plasmid	Spec	5	[246]
pOGG200	pL0M-*phlD*	Spec	5	Barney Geddes
pOGG201	pL0M-*phlE*	Spec	5	Barney Geddes
pOGG199	pL0M-*phlACB*	Spec	5	Barney Geddes
pOGG0121	pL0M-P*J23106*	Spec	5	Barney Geddes
pOGG208	pL1M-P*J23106::phlACB*	Amp	5	Barney Geddes
pOGG215	pL1M-P*J23106::phlD*	Amp	5	Barney Geddes
pOGG160	pL0M-T-F2	Spec	5	Barney Geddes
pOGG214	pL1M-P*J23106::phlE*	Amp	5	Barney Geddes
pOPS0910	pRK2 constitutive 2,4-DAPG biosynthesis plasmid	Kan/Neo	5	Barney Geddes
pOGG098	pL0M-P*mocB*	Spec	5	Barney Geddes
pOGG208	pL1M-F1-P*mocB::phlACB*	Amp	5	Barney Geddes
pOGG209	pL1M-F2-P*mocB::phlD*	Amp	5	Barney Geddes
pOGG213	pL1M-F3-P*mocB::phlE*	Amp	5	Barney Geddes
pOPS0909	pRK2 SI inducible 2,4-DAPG biosynthesis plasmid	Kan/Neo	5	Barney Geddes
pOPS1907	P*phlA::gfp* pBBR1 plasmid	Kan/Neo	5	This work
pOPS1847	SI inducible 2,4-DAPG plasmid with *PmocB::mocR*	Kan/Neo	5	This work
pOPS2093	RK2 SI inducible 2,4-DAPG plasmid	Tet	5	This work
pOPS2095	pBBR1 SI inducible 2,4-DAPG plasmid	Kan/Neo	5	This work
pOPS2094	RK2 SI inducible *phlACB* with PJ*23106::phlD-phlE*	Tet	5	This work
pOPS2096	pBBR1 SI inducible *phlACB* with PJ*23106::phlD-phlE*	Kan/Neo	5	This work
pOGG137	pL0M *phlF*-P*phlA*	Spec	5	Barney Geddes

pOGG002	pL0M *luxCDABE*	Spec	5	[246]
pOPS1862	2,4-DAPG inducible *luxCDABE*	Gent	5	This work
pOPS2097	RK2 SI inducible WSM1497 *traR-traI*	Tet	5	This work
pOPS1917	RK2 P*lux::luxCDABE*	Tet	5	This work
pMR69	luxR-P*lux* module	Kan/Neo	5	[99]
pOPS2158	AHL inducible *lux* plasmid	Tet	5	This work
pOPS2098	P*mocB::traR-traI*	Tet	5	This work
pOPS2099	SI inducible RBS_{28}-*traR-traI*	Tet	5	This work
pOPS2100	P*mocB::traI* AHL plasmid	Tet	5	This work
pOPS2155	AHL biosensor *luxR-Plux::gfp*	Tet	5	This work
pOPS1487	PK19mobSacB-R6K vector	Kan/Neo	5	Timothy Haskett
pOPS2154	2,4-DAPG inducible *E. radicincitans nifA* controller	Tet	5	This work
pOPS2153	AHL inducible *E. radicincitans nifA* controller	Tet	5	This work

Table 2.3 Primers Used in This Study

Name	Sequence	Chapter
Oxp2115	CTAGAGCTAGCATTGTACCAAGGGCTGAGCTAGCTATAAAAAAAAAAAAACCCCGCCCTGTCAGGGGCGGGGTTTTTTTTTTCAGCAACCACGTGGAGC	3
Oxp1868	TTTGAATTCTCTTACGACCTGACCCGCTA	3
Oxp1431	TAGCCCTTAGTGACTCTAATACGACTCACTATTGGGAGATTCAGTGCTTGCGGCGTTT	3
Oxp1432	AGTTTCAGGGAAAGAACTGAGGCGGATGCCACACAGGAT	3
Oxp2114	TGATTACGCCAAGCTACGGTGCAGGATCTTGGC	3
Oxp1856	AAAGCTCAGAAGCGCTTGATCTCGAT	3
Oxp1869	TAGCCCTTAGTGACTCTAATACGACT	3
Oxp1336	TTTTGAAGACAAAATGAGCACAAAAAAGAAACCATTAACACAAG	3
Oxp2319	TGTTGGATTGCGCCGGCACTGAAGAGTGGAGGAGAAAGAATGCGGAATGCTCGCAGCCGCTTTTGAGGAGGCGAGAATGCCAATGACCGACGC	3
Oxp1438	CTTTCTCTAGTAGCTAGCCCAAAAAAACGGGTATGGAGAAGGTCATCTTCGCCTCTCAGC	3
Oxp4009	CCGCGCCCTCTCAGAAGCGCTTGATGTCG	3
Oxp4010	GCGCTTCTGAGAGGGCGCGGCGCGCGAG	3
Oxp1438	CTTTCTCTAGTAGCTAGCCCAAAAAAACGGGTATGGAGAAGGTCATCTTCGCCTCTCAGC	3
Oxp3346	CCGAGATCCTCGGCCATCAT	3
Oxp1283	TTTGAAGACAATGTCCAAGGGCGAGTTCC	3
Oxp2960	CCGGGTACCGAGCTCGAATTGAATTCCCTGGCAGTTTATGGC	3
Oxp2961	AAAACGACGGCCAGTGAATTGGCAGCCTCGCTCGATG	3

Oxp4186	GTCGACTCTAGAGGATCCCCTCATCACCACCGCATGGC	3
Oxp4187	TGAATTCGAGCTCGGTACCCAGCGGGTTGGTGAAGGCG	3
Oxp4182	GTCGACTCTAGAGGATCCCCACCGTGGACGAGACGCGC	3
Oxp4183	CGTCGGCGTCGGCCATCACGATCTTCATGGATCG	3
Oxp4184	CGTGATGGCCGACGCCGACGCGCTCTGA	3
Oxp4185	TGAATTCGAGCTCGGTACCCTGGCGATGAAGGTGTTCATCATGG	3
Oxp0284	GGCGGATTTGTCCTACT	4
Oxp0283	AGCGTTCTGAACAAATCC	4
Oxp2750	TATTTGATGCCTGCCGAATTTAGCCCTTAGTGACTCTAATACGACT	4
Oxp2751	AAAACGACGGCCAGTGAATTCCGTTTAGAGGCCCCAATC	4
Oxp4954	TTTTGAAGCTAATTCGAGATCATGCATAATGCCGAATTCGTAGCCCTTAGTGACTCTAATACG	4
Oxp4953	TGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGGTTATTTGTATAGTTCATCCATGCCATGTG	4
Oxp5878	AAAGGTGAGACGCATCCTGTGTGGCATC	4
Oxp5879	GCCCCAATCTAGATCTTTGGCACGACGTTGTAAAACGACG	4
Oxp4118	AAAAAACGGGTATGGAGAAGTTAGAGCTTTCTCACGTCCACA	4
Oxp4935	AAGAGTGGAGGAGAAAGAAGAGTGCCCTGTCTGTACCTTC	4
Oxp5546	CGTCGCTTGACCAAATTGGC	4
Oxp5547	GAGAAGGCTCTCAGACAGCG	4
Oxp5822	AAGAGTGGAGGAGAAAGAAGGCGGCCGCCTACTCTAGAATTAAAGAGGAGAAATTAAC	4
Oxp5823	GCCAGTGGGGTATGGAGAAGTTACTTGTACAGCTCGTCCATG	4
Oxp1339	TTTTGAAGACAAAGATTGCATGTAACGCGTGCGA	5
Oxp1340	TTTTGAAGACAAATCTTCTAAAACTTTTGTGATGGTTGATCGG	5
Oxp1338	TTTTGAAGACAAAATGTTTGGACCGGATTTCCCGTTT	5
Oxp1341	TTTTGAAGACAAAAGCTTATTAGCCTTTTAACACTTTTTCGATCAAA	5
Oxp1137	TTTTGAAGACAAAATGAATAAAGTAGGAATTGTGAGCTA	5
Oxp1138	TTTGAAGACAAGAGGACGTCATTACCTTGGGCGT	5
Oxp1139	TTTGAAGACAAAGGACCAGACTCGCCGC	5
Oxp1140	TTTGAAGACAAACACGACATAATCGAAATCGCCG	5
Oxp1141	TTTTGAAGACAAAAGCTTATTTCACCAATACAAACTTATAGGC	5
Oxp1142	TTTGAAGACAAGTGTTCCAACAGAACCTAGTGTC	5
Oxp1143	TTTGAAGACAATCCTCAAGGCCGATGCTGTTCTT	5
Oxp1144	TTTGAAGACAACCTCATCGGGTTGCAGGACG	5
Oxp2074	TGTGAAGACAATGCCGAATTC	5
Oxp2075	TTTGAAGACAACAGAATCAAAGCAGCGGGCTGGAGAGCCAAAAAACTTGACTGTTGTTATCGAAAGAGCGGGATGTAGCGGGCGCCTAGTTTGTTATTTCACCAATACAAACTTATAGGC	5
Oxp5213	GTCGACTCTAGAGGATCCCCTAGCTATAAAAAAAAAAAAACCCC	5

Oxp5214	TGAATTCGAGCTCGGTACCCTTCTTTCTCCTCCACTCTTC	5
Oxp4238	ATAATGCCGAATTCGGATCCCTCGAGTGCCTTGTCTTCTGCAGC TGGCACGACAGGT	5
Oxp4239	TCTATCAACAGGAGTCCAAGTCCCTTGTCTTCGTTCACAGCTTG TCTGTAAGCGG	5
Oxp5635	AAGAGTGGAGGAGAAAGAAGATGAATAAAGTAGGAATTGTGA GC	5
Oxp2521	TTTTTCTAGATTATCCCACCGCTGCTAACG	5
Oxp2581	GGAGGAGAAAGAAGGATCATGAATAAAGTAGGAATTGTGAGC T	5
Oxp2582	GGGTATGGAGAAGGATCTCACTTATCCTCCAGGGTCA	5
Oxp5633	AAGAGTGGAGGAGAAAGAAGCAACGGCACGGAGGTTTATG	5
Oxp5634	GCCAGTGGGGTATGGAGAAGTTAAGCGTATGCCGGCAG	5
Oxp5713	GATCCGGAGAAACTAGTAGCTTAATTTTTAAAGTATGGGCAAT C	5
Oxp5714	TCCTCTTTAATTCTAGATGCTTTATTCGAAAGTAACAAACC	5
Oxp5757	AAGAGTGGAGGAGAAAGAAGTCTAGAGCTAATCTTCGCGTACT AAGAACGCAAGTAATGCATCGCGTGTTTGAAAATTTC	5
Oxp5634	AAGAGTGGAGGAGAAAGAAGTCTAGAGCTAATCTTCGCGTACT AAGAACGCAAGTAATGCATCGCGTGTTTGAAAATTTC	5
Oxp5898	AAGAGTGGAGGAGAAAGAAGATCCTGCACGGAGGCGAC	5
Oxp5899	GCCAGTGGGGTATGGAGAAGTTAAGCGTATGCCGGCAGG	5
Oxp5882	TGCCTGCCGAATTCGGATCCTGCTCATGTTTGACAGCTTATC	5
Oxp5883	TCTATCAACAGGAGTCCAAGAAAAAGGCCATCCGTCAG	5
Oxp5910	GTCGACTCTAGAGGATCCCCGCGGCTGTGCCTTATTCC	5
Oxp5911	GCTGAGTCATCACAAACTCCTGTGGTCAAC	5
Oxp5912	GGAGTTTGTGATGACTCAGCGAACCGAG	5
Oxp5913	TGAATTCGAGCTCGGTACCCCCATTTCGCCTTCCTGCAAG	5
Oxp5914	GTCGACTCTAGAGGATCCCCCGGCGGTTGTGGTGGTGG	5
Oxp5915	AAAACCTGCGAGTGAGCCTCCGGTCAGTG	5
Oxp5916	GAGGCTCACTCGCAGGTTTTTGTGACATTCGGC	5
Oxp5917	TGAATTCGAGCTCGGTACCCCAGCTCGCCGCAACGCTC	5
Oxp5918	GTCGACTCTAGAGGATCCCCGCGGCTGTGCCTTATTCC	5
Oxp5919	AAAACCTGCGCACAAACTCCTGTGGTCAAC	5
Oxp5920	GGAGTTTGTGCGCAGGTTTTTGTGACATTCGGC	5
Oxp5921	TGAATTCGAGCTCGGTACCCCAGCTCGCCGCAACGCTC	5
Oxp5922	CAAACAGCGCCACGGTTTTA	5
Oxp5923	CAGATAAAGGTTCGCGCTGC	5
Oxp6049	ATCGTTAAGGTTACTAGAGTTCACTGACCGGAGGCTCAC	5
Oxp6050	TTAATTTCTCCTCTTTAATTCTACATTCTCGGCATGGTAATATCC ATC	5
Oxp6053	TTTGTTACTTTCGAATAAATTCACTGACCGGAGGCTCAC	5
Oxp6054	TTAATTTCTCCTCTTTAATTCTACATTCTCGGCATGGTAATATCC ATC	5
Oxp2480	CTTTGATCTCCGGGTAGCC	5
Oxp4046	CAGTCACGACGTTGTAAAACGA	5

2.2 Molecular Techniques

2.2.1 DNA Isolation

Plasmid DNA was isolated from bacterial cultures using the Monarch Plasmid Purification Kit (New England Biolabs). To purify DNA following a restriction digest or PCR, the GeneJET PCR Purification Kit (Thermo Fisher) was used. Genomic DNA was isolated from *S. meliloti, E. radicincitans,* and *Mesorhizobium* WSM1497 using PrepMan Ultra (Thermo Fisher) sample preparation reagent. All purifications were performed according to the respective manufacturer's instructions.

2.2.2 Primer Design

Primers for PCR and sequencing reactions were designed using Geneious R10 software. Oligonucleotides were synthesised by Eurofins Genomic. Sequencing of cloned plasmid products was carried out by Source Bioscience using Sanger sequencing. Full plasmid sequencing was performed by Plasmidsaurus by long-read library sequencing using Oxford Nanopore R10.4.1 flow cells.

2.2.3 DNA PCR Amplification

High fidelity amplification of DNA products for use in cloning reactions was performed using the Q5 DNA Polymerase (New England Biolabs). PCR mapping reactions were performed using OneTaq DNA Polymerase (New England Biolabs). Primer melting temperatures were determined by the New England Biolabs T_m calculator. A Verti thermocycler (Applied Biosystems) was used for all reactions, with reactions and conditions set according to the manufacturer's instructions. PCR products for downstream cloning were purified using a GeneJET PCR purification kit (Thermo Fisher) according to manufacturer's instructions.

2.2.4 Gel Electrophoresis

Plasmid and PCR products were analysed using Gel electrophoresis to separate products based on size. Gels consisted of 0.9% agarose (w/v) with SYBR Safe DNA dye (Invitrogen) added to a 1:10000 ratio in TAE buffer (500 mM TRIS acetate, 1 mM EDTA) and were run at 120mV for 30 minutes. Gels were subsequently visualised using a GelDoc EZ system (BioRad).

2.2.5 DNA Sequencing

Sanger sequencing of plasmids and PCR fragments was carried out by Eurofins Genomics. Primers were designed using Geneious version 10.0.9 and premixed with samples according to the sequencing service's guidelines.

2.2.6 Restriction Digest

Plasmid restriction digests were carried out using restriction endonucleases and their respective buffers (NEB or Thermo Fisher) according to the manufacturers' protocols.

2.2.7 Golden Gate Cloning

Domesticated genetic modules PU module (promoter with the ribosome binding site), SC module (open reading frame), and the T module (terminator) were used to construct transcriptional units in level 1 plasmids via Golden gate cloning. The genetic modules when digested by type II restriction enzymes have complementary sticky-ends which result in the DNA fragments assembling in the correct order in a vector. BsaI was used for the assembly of the expression cassette into level 1 plasmids, and BpiI for assembly of level 1 modules into level 2 plasmids. Golden Gate reactions were carried out in 15 µL reactions containing 1.5 µL T4 DNA ligase buffer, 1.5 µL BSA diluted 10x, 1 µL BsaI, and 1 µL T4 DNA ligase (Thermo Scientific), as well as DNA equal to 40 fmol for each Golden Gate part. Assembly reactions

were performed in a thermocycler with the following protocol: 25 cycles of 3 minutes at 37°C and 4 minutes at 16°C, followed by 5 minutes at 50°C then 5 minutes at 80°C.

2.3 Media and Bacterial Growth

E. coli and *E. radicincitans* strains were grown at 37°C in Luria Bertani broth (LB) (10g L^{-1} tryptone, 5g L^{-1} yeast extract, 5g L^{-1} NaCl). Cultures were shaken at 200 rpm unless otherwise stated. For growth on solid media, agar was added to LB broth at 1.5% (w/v) prior to autoclaving. Rhizobial species *A. caulinodans* and *S. meliloti* were grown on tryptone yeast (TY) media (5 g L^{-1} tryptone, 3 g L^{-1} yeast extract, 6 mM $CaCl_2$). For growth on solid media, agar was added to 1.5% (w/v) before autoclaving.

When a minimal growth media was required, rhizobacteria were grown in Universal Minimal Salts (UMS). UMS contains 0.5 mM K_2HPO_4, 0.5 g L^{-1} $MgSO_4.7H_2O$, 0.2 g L^{-1} NaCl, 4.19 g L^{-1} MOPS, adjusted to pH 7.0 and autoclaved. Following autoclaving 1L of media was supplemented with 1 mL of iron stock solution (12 g L^{-1} $FeSO_4.7H_2O$), 1 mL of calcium stock solution (75 g L^{-1} $CaCl_2.2H_2O$) and 1 mL of a stock solution containing 1 g L^{-1} thiamine hydrochloride, 2 g L^{-1} D-Pantothenic acid calcium salt and 100 mg L^{-1} biotin. Growth in UMS was supplemented with filter-sterilised 20 mM succinic acid (succinate), 10 mM NH_4Cl, and 1 µL of 1000X vitamin solution (containing 0.375 g L^{-1} EDTA-Na_2, 0.16 g L^{-1} $ZnSO_4.7H_2O$, 0.2 g L^{-1}, $NaMoO_4$, 0.25 g L^{-1} H_3BO_3, 0.2 g L^{-1} $MnSO_4.4H_2O$, 0.02 g L^{-1} $CuSO_4.5H_2O$, 1 g L^{-1} $CoCl_2.6H_2O$), For growth on solid media, agar was added to 1.5% (w/v) before autoclaving. For *E. coli* M9 media was used, containing 6 g L^{-1} Na_2HPO_4, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} NaCl, 1 g L^{-1} NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.2% w/v glucose. *A. caulinodans* was grown at 37°C, with 200 rpm shaking for liquid media. *A. caulinodans* is auxotrophic for nicotinate, so 30 µM nicotinic acid was added to UMS growth medium for this strain. *S. meliloti* was grown at 28°C, with 200 rpm shaking for liquid media. All bacterial strains were stored

by adding liquid bacterial culture to a final concentration of 20% (v/v) glycerol before freezing in liquid nitrogen for storage at -80°C.

2.3.1 Antibiotics

Appropriate antibiotics were added to media to final working concentrations listed below. *A. caulinodans* ORS571 has chromosomal resistance to nitrofurantoin and ampicillin, the latter was used as a selection antibiotic. *S. meliloti* CL150 has chromosomal resistance to streptomycin which was used as a selection antibiotic. *E. radicincitans* was selected for on nitrofurantoin.

Table 2.4 Antibiotics Used in This Study. Units in µg ml^{-1}

	E. coli	*A. caulinodans*	*S. meliloti*	*E. radicincitans*
Ampicillin	100	100	50	50
Gentamycin	20	20	20	20
Kanamycin	20	20	20	20
Neomycin	-	80	80	80
Streptomycin	250	250	250	250
Tetracycline	10	5	5	5
Nitrofurantoin	-	5	-	5

2.3.2 Microaerobic Growth

A. caulinodans ORS571 and *E. radicincitans* were grown under microaerobic conditions in an atmospheric regulatory cabinet at 3% (v/v) oxygen to facilitate nitrogen fixation. Microaerobic assays were performed in gas tight universal tubes with rubber gas sampling apertures (VWR, Pennsylvania, USA).

2.3.3 Growth Curves

Growth curves were performed in triplicate by streaking single colonies onto 10 mL TY agar slopes and incubating for 3-days prior to three washes in PBS and inoculation at OD_{600nm} 0.01 into 500 µL UMS media in 24-well plates. The OD_{600nm} was monitored at 20

minute intervals in an Omega FLUOstar plate reader set to shake cultures at 700 rpm at 37°C until stationary phase was reached.

2.4 Bacterial Transformation

2.4.1 Thermal Competent *E. coli*

Chemically competent *E. coli* DH5α cells were used for to store and confirm DNA plasmids. Competent *E. coli* were produced by inoculating 500 mL of LB from a single colony and incubating for 3 hours until a culture OD_{600} of 0.3-0.4 was achieved. The mid-log culture was incubated on ice for 10 minutes before cells were collected by centrifugation at 4000 *g* at 4°C for 10 minutes. The pelleted cells were resuspended and washed in 20 mL pre-chilled 0.1M $CaCl_2$ and 15% glycerol (v/v) before further incubation on ice for 30 minutes. Cells were once again pelleted and the wash solution removed before a second wash in 20 mL 0.1M $CaCl_2$ 15% glycerol (v/v) and incubation on ice for 30 minutes. Cells were subsequently aliquoted in 50 µL volumes and frozen in liquid nitrogen before long-term storage at -80°C. Competent *E. coli* EC100D Pir+ strains for maintenance and transfer of R6K origin plasmids were prepared using the same method.

2.4.2 Thermal Transformation *E. coli*

For transformation of plasmids into *E. coli* strains, plasmids suspended in TE buffer at a concentration of 50-200 ng μL^{-1} were added to a thawed 50 µL aliquot of competent *E. coli* and incubated on ice for 30 minutes. The cell and plasmid mix was heat-shocked at 42°C in a water-bath before transfer back to ice for a further 2 minute incubation. The cell culture was recovered in 900 µL of SOC media (Melford) and incubated with shaking at 37°C for 1 hour. Successfully transformed cells were selected by plating serial dilutions on LB agar plates containing appropriate antibiotic and incubating overnight at 37°C.

2.4.3 Rhizobacteria Conjugation

For the conjugation of plasmids into rhizobial strains, triparental mating was used, whereby *E. coli* carrying the helper plasmid pRK2013 were incubated with the desired rhizobial recipient and DH5α plasmid doner strain. Prior to transformation, rhizobia were grown on TY agar slopes at 37°C for 48 hours. *E. coli* DH5α plasmid doner and helper strains were inoculated in 10 mL LB broth with antibiotics and incubated with shaking overnight at 37°C. Overnight *E. coli* cultures were sub-cultured by 1:10 dilution into fresh LB containing appropriate antibiotics and grown for a further 3-4 hours at 37°C until the culture reached mid-log growth stage, OD_{600} ~0.5. *E. coli* cultures were subsequently centrifuged at 4000 *g* for 10 minutes and washed with 1 mL of TY medium three times in order to remove traces of antibiotics. The rhizobacteria slope was washed with 2 mL of TY and the cells pelleted by centrifugation before resuspension in 400 µL of TY. Recipient rhizobacteria (200 µL) was mixed with 200 µL of both donor *E. coli* and pRK2013 helper *E. coli* strains. The bacterial suspension was centrifuged at 4000 *g* for 5 minutes and the pellet resuspended in 50 µL of TY medium and spotted on a non-selective TY plate, this process was repeated to produce an adjacent negative control conjugation spot containing only the rhizobacteria recipient strain and *E. coli* helper. The conjugation plate was incubated at 28°C for *S. meliloti* and 37°C for *A. caulinodans* and *E. radicincitans* overnight. A sterile loop was then used to streak bacteria from the conjugation spot onto a fresh TY plate containing appropriate antibiotics and incubated for 2 days at the optimal temperature for the rhizobial recipient. Antibiotics on the outgrowth plate were used to select against *E. coli*, nitrofurantoin (5 µg mL^{-1}) was used to select against *E. coli* when conjugating into *E. radicincitans*, ampicillin (100 µg mL^{-1}) for *A. caulinodans* ORS571, and streptomycin (50 µg mL^{-1}) for *S. meliloti*.

2.4.4 Mini-Tn7 Integration

Gene constructs were integrated into the chromosome of bacteria by mini-Tn7 integration. Integration requires the pTNS3 plasmid carrying the transposases *tnsABCD* which insert the transcriptional unit into the region downstream of the *glmS* gene. Insertion of pUC18R6K-mini-Tn7T-Km plasmids was performed in a similar manner to tri-parental conjugation, with EC100D *pir+ E. coli* as plasmid donor alongside *E. coli* containing pTNS3 and pRK2013 helper plasmids. Constructs were integrated downstream of *glmS* in *E. radicincitans* and an engineered landing pad site in *S. meliloti* OPS1835 with inserted Tn7 *attB* sites amplified from *R. leguminosarum*[249].

2.4.5 Markerless Deletion

Markerless gene deletions were performed with pK19mobSacB plasmids with 1Kb of flanking chromosomal regions inserted into the restriction digested plasmid by HiFi cloning. The resulting plasmid was transformed into ECD1000 Pir+ *E. coli* for maintenance. The plasmid was mobilised into the recipient bacterial strain by tri-parental conjugation to generate the mutation. Single crossover of the suicide plasmid with the cloned genomic regions of interest can be screened by colony PCR. Single crossover mutants were grown in liquid culture and inoculated on agar media with 10% v/v sucrose. Successful double crossover mutants were screened for deletion of the desired gene by colony PCR with genomic primers.

2.5 Bacterial Assays

2.5.1 Fluorescent Reporter Expression Analysis

The induction of reporter constructs carrying GFP or mCherry fluorescent proteins in all bacteria tested was measured using a CLARIOStar Omega microplate reader (BMG). A fluorescent reporter assay has been developed in this lab, whereby single bacterial colonies

totalling three biological replicates with the desired fluorescent reporter were selected and grown in TY with appropriate antibiotic overnight at the optimal culture growth temperature. Cultures were pelleted at 5000*g* for 10 minutes and resuspended in UMS with 1:1000 vitamins, 20 mM succinate, 10 mM NH_4Cl to a final OD_{600} of 0.3, 500 µL of biological replicates were added to each well in a 96- deep well plate. At this point if an inducer was required it was added to the desired concentration and the culture incubated for 18 hours overnight at the optimal growing temperature with shaking at 200 rpm. 200 µL of each test culture was transferred to a black 96-well microplate with a transparent bottom (Vision Plate 24, 4titude). Fluorescence intensity was measured in the microplate reader with GFP fluorescence measured at excitation 485 nm and emission 520 nm, whilst mCherry fluorescence was measured with excitation at 560 nm and emission at 590 nm, gain was set for both at 1250. The OD_{600} of cultures was also measured to allow normalisation of fluorescence to the optical density, and thereby the cell number, of the culture giving a relative fluorescence unit (RFU) normalised to the background fluorescence of the media. Both fluorescence and growth data were processed using the MARS data analysis software. For standard curves of biosensor induction by inducers, logistic models were fitted using the AAT Quest Graph Four Parameter Logistic (4PL) Curve Calculator.

2.5.2 ORS571 Acetylene Reduction Assay

Strains were streaked on TYA supplemented with 5 µM SI and appropriate antibiotics. A single colony showing good GFP induction under transillumination was selected to inoculate an UMA slope with a carbon and nitrogen source for overnight incubation to allow growth of the colony. The cultures were washed, resuspended, and diluted in 2 mL UMS to OD_{600} 0.4 with 10 mM rhizopine inducer with or without 10 mM NH_4Cl at 3% oxygen. Cultures were then incubated for 9 hours at 30°C with vigorous shaking before replacing 10% of the headspace atmosphere with acetylene before a further three hour incubation followed by

measurements of the ethylene/acetylene ratio using injection of 1 mL of headspace atmosphere in a Clarus 480 gas chromatograph (Perkin Elmer).

2.5.3 *E. radicincitans* Acetylene Reduction Assay

DSM16656 strains were grown overnight in LB followed by centrifugation and washing thrice in UMS. Cultures were resuspended in UMS with 10 mM glucose and vitamin solution to OD 0.4, with 10 mM ammonium chloride added to the high nitrogen assay replicates. For relay signalling, cultures were mixed 1:1 with induced *S. meliloti*. Cultures were incubated at 3% oxygen for 12 hours in the presence of 10% headspace acetylene before measurement of N_2 fixation via ARA using a Clarus 480 gas chromatograph (Perkin Elmer).

2.5.4 BCA Assay

A bicinchoninic acid assay was used to determine the protein concentration of the induced cultures. A standard curve was prepared of 0, 02, 0.4, 0.6, 0.8 and 1mg mL^{-1} bovine serum album with distilled water in a volume of 25 µL. 1 mL of induced cultures was homogenised by two rounds of 30 second shaking in a Ribolyser (Thermo Fisher) and the supernatant extracted. 25 µL of supernatant was added to 200 µL working reagent (1 mL 4% Cu_2SO_4 stock solution in 50 mL bicinchoninic acid) in wells of a microplate. Samples were incubated for 30 minutes at 37°C before measurement of absorbance at 562 nm using a FLUOstar Omega plate reader. The standard curve of known protein concentrations was used to estimate the protein content of rhizobia cultures.

2.5.5 Ammonia Indophenol Determination

A. caulinodans cultures were grown for 24 hours in N-free UMS containing 20 mM succinate and 10 µM rhizopine with 3% O_2 headspace. Culture supernatants were collected by centrifugation and diluted 1:2 to 750 µL and mixed with 150 µL sodium phenate, 225 µL of 0.66 mM sodium nitroprusside dihydrate and 225 µL of 1.5% sodium hypochlorite. Samples

were incubated for 1 hour before quantifying the resulting indophenol-blue by spectrophotometer at 630 nm.

2.5.6 IAA Measurement by Salkowski's Reagent

Bacterial cultures were grown in triplicate in TY with and without tryptophan for 18 hours. Bacterial cultures were resuspended in PBS and diluted to OD_{6000nm} 1.0, 100 µL of bacteria culture supernatant was collected and combined with 200 µL of Salkowski reagent (0.5 M ferric chloride and 35% perchloric acid) in triplicate. The plate was covered and incubated in the dark for 30 minutes, due to the light sensitivity of IAA, before measurement of absorbance at 530 nm using a microplate reader. The absorbance value produced from incubation with just the culture media was subtracted from measurements to account for any background indole reaction.

2.5.7 2,4-DAPG Production Assay

2,4-DAPG biosynthesis plasmids were grown to an OD_{600} of 0.6 in 10 mL of TY media at 37˚C with 200 rpm shaking and the relevant antibiotics and 10 µM SI for inducible strain. Cultures were then centrifuged at 4500 g for 10 minutes at 4˚C to pellet the cells and debris and the supernatant collected. *E. coli* cells containing the 2,4-DAPG biosensor pOPS1907 were grown overnight in LB media supplemented with antibiotic. *E. coli* cultures were centrifuged at 4500 g for 10 minutes at 4˚C to pellet the cells and resuspended to OD_{600} 0.1 in M9 media. To measure induction, 400 µL of the resuspended cultures was added to a 96-deep well plate alongside 100 µL of CL150 strain culture supernatant. Combined cultures were incubated for 18 hours and then 100 µL transferred to a transparent 96-well plate for GFP fluorescence and OD_{600} plate reader measurements. Subsequent fluorescence results were expressed as RFU and measured with three biological replicates for each CL150 2,4-DAPG biosynthesis strain.

Induction from the *E. coli* biosensor was normalised by subtracting the background induction measured from the addition of supernatant from WT CL150.

2.5.8 AHL Lux Induction

96-well plates were poured with 180 µL of TY with 1.5% agar, with or without 10 µM SI. Colonies of *S. meliloti* strains were streaked on TYA slopes with appropriate antibiotics and grown for 3 days at 28°C. After 3 days the culture was washed off the slope in 2 mL TY, centrifuged, and resuspended in 200 µL TY. The dense resuspension was used to spot 50 µL of culture (~ 10^9 cells) onto the centre of the 1.5% TYA plate surface and incubated for 48 hours. Subsequently, the microtiter plates and *Sinorhizobium* spot was covered with 130 µL of 1:1 mixture of stationary phase *E. coli* Lux biosensor pOPS2158 and LB with 1.5% (w/v) agarose. The *E. coli* and LB agarose mixture was kept in a water bath at 50°C, ensuring the agarose remained melted for pouring whilst allowing for survival of *E. coli* cultures. After incubating the plates overnight at 28°C, the luminescence of the *E. coli* bioreporter was measured using the GloMax luminometer with an integration period of 100 mS. The luminosity (cps) was divided by the area of the plate measured (mm) and normalised to luminosity when *E. coli* was incubated with WT CL150. Larger petri-dish plates using this method were imaged using the NightOWL II LB 983 imaging system (Berthold).

2.6 Plant Growth

2.6.1 *H vulgare* Seed Preparation

Golden promise barley seeds, or T2 *RhiP* lines with integrated rhizopine biosynthesis plasmid pEC12811 expressing *idhA* and *mosB* genes under the control of *Z. mays* ubiquitin1 promoter and *O. sativa* ubiquitin 1 promoter respectively[227], were surface sterilised by washing in 70% ethanol for 2 minutes followed by decanting the ethanol and washing in 7% NaOCl for a further 2 minutes followed by rinsing in an abundance of sterile dH_2O. Seeds were germinated

by placing the sterile seeds onto 1% water-agar plates and incubating in the dark for 2 days at 28°C. Only seeds with a radicle > 1cm were selected for *in situ* assays. Germinated seeds were placed in a 100 mL Schott bottle with 50 g industrial sand and 25 mL N-free rooting solution ($CaCl_2 \cdot 2H_2O$ 2.67 mM, KCl 276 µM, $MgSO_4 \cdot 7H_2O$ 2.13 mM, Fe EDTA 26.67 µM, H_3BO_3 93.33 µM, $MnCl_2 \cdot 4H_2O$ 24 µM, $ZnCl_2$ 2.13 µM, $Na_2MoO_4 \cdot 2H_2O$ 1.33 µM, $CuSO_4 \cdot 5H_2O$ 0.8 µM, KH_2PO_4 1.33 g L^{-1}, Na_2HPO_4 1.52 g L^{-1}). Seeds were inoculated with washed bacterial suspensions in UMS with 10 mM glucose and 10 mM ammonium chloride at OD_{600} 0.1. Schott bottles were covered with sterile cling film and placed in a growth chamber with 23°C 16 hr light / 21°C 8 hr dark cycles.

2.6.2 *In Situ* Nitrogen Fixation in Barley

At 6 days post inoculation, Schott bottles were placed in a controlled atmosphere cabinet at 3% O_2 and incubated for 1 hour. The bottles were sealed using screw caps with a rubber septum and the headspace replaced with 10% acetylene. Ethylene production was measured after 12 hours incubation using a PerkinElmer, Clarus 480 gas chromatograph.

2.6.3 *E. radicincitans In Planta* ARA

Acetylene reduction assay was performed according to the method published by Haskett *et al,* 2021[249]. In short, a single colony of CL150 strains were grown on TY agar for 5 days before inoculation, whilst *E. radicincitans* strains were grown overnight in LB. The barley seeds were surface sterilized in 70% ethanol and 7% NaOCl followed by germination on 0.9% agar for 2 days. Germinated seeds were placed in a sterilized Schott bottle filled with 50g of sand with 25 mL of rooting solution with 1 mM KNO_3. To initiate the assay, CL150 relay strains were washed from agar slopes and resuspended to OD_{600} of 0.1 in UMS media before inoculation of 2 mL of bacterial solution around the seedling. The barley with CL150 were incubated for 5 days in a growth chamber with 23°C 16 hr light / 21°C 8 hr dark cycle before

addition of 2 mL of OD_{600} 0.1 *E. radicincitans nifA* strains suspended in UMS with 10 mM glucose and 10 mM ammonium chloride. The plants were then incubated overnight before placing the Schott bottle in a 3% O_2 atmosphere growth cabinet for 1-2 hours. The bottles were then sealed and 10% of the headspace atmosphere replaced with acetylene, with measurements of ethylene production taken after 12 hours using a PerkinElmer, Clarus 480 gas chromatograph.

2.6.4 Flow Cytometry

For single cell gene expression analysis of bacterial cells from plant associated wash fractions were analysed by flow cytometry[249]. Bacteria occupying the root-associated (RA) fraction of barley roots were isolated by excising the root below the seed and vortexing the root for 1 minute in 20 mL PBS to remove loosely attached bacteria prior to crushing the roots with a sterile mortar and pestle and resuspending the crushed root in 5 mL of PBS. The rhizosphere (RS) fraction was isolated by rinsing bacteria from the remaining sand with 20 mL of PBS and vortexing for 1 minute. All samples were passed through a 40 μm FLOMI cell strainer and RS samples were diluted 1:10 prior to analysis by flow-cytometry. Culture fluorescence was measured using an Amnis Cellstream flow-cytometer via autosampler, and at least 50,000 events were counted for each sample. GFP and mCherry were excited with 488nm and 561nm lasers, respectively. Based on prior analysis in this lab, bacterial singlets were gated into fluorescent populations using the CellStream Analysis 1.3.382 software. Bacterial singlets were gated based on the forward scatter (FSC) and side scatter (SSC). Singlets exhibiting mCherry fluorescence (detected at 611/31 nm) above 5000 a.u were considered as the mCherry+ bacterial population for analysis of P*mocB* expression. mCherry+ bacteria exhibiting GFP fluorescence (detected at 528/46 nm) above the mean 99th percentile of fluorescence determined for noninduced bacterial populations colonising WT barley roots were defined as GFP+.

2.6.5 *Medicago Truncatula* Growth

2.6.5.1 Seed Germination

M. truncatula R108 or EC52754-pL2B-Basta-IDH-StsC-eGFP SI producing lines[227] shell pods were cracked and the extracted seeds lightly scarified with sandpaper before sterilisation in 20% v/v NaClO solution for 2 minutes. Seeds were washed 10 times with 50 mL sterile ultrapure water and left to imbibe for 48 hours at 4°C before germinating the seeds on 1.2% agar H_2O square plate at 25°C for 3 days until the roots were > 3cm long.

2.6.5.2 Microcosms

Microcosms were constructed from standard, round 100 mm diameter, 15 mm deep laboratory plates filled with ~70 mL FP agar nutrient media. The roots of *M. truncatula* were grown vertically down the agar surface, the top of plates were notched with a hot scalpel to allow shoot growth. Plants were inoculated with 100 µL an overnight culture of *S. meliloti* diluted to OD_{600} 0.05 in sterile ultrapure water. The individual microcosms were placed in foil wrapped stacks in a lighted growth chamber at 22°C and 150 µmol m^{-2} s^{-1} light on a 16 hr light / 8 hr dark cycle.

2.6.5.3 Single Pot Growth

Plastic pots were double stacked with a paper barrier sandwiched between the layers and filled with 50 mL fine-grade sand watered with 10 mL plant rooting solution ($CaCl_2.2H_20$ 1 mM, KCl, 100 µM , $MgSO_4.7H_2O$ 800 µM, Fe EDTA 10 µM, H_3BO_4 35 µM, ZnCl2 0.8 µM, $Na_2MoO_4.2H_2O$ 0.5 µM, $CuSO_4.5H_2O$ 0.3 µM, KH_2PO_4 25 g L^{-1}, Na_2HPO_4 28.4 g L^{-1}) supplemented with 1 mM KNO_3. Pots were autoclaved and individual pots planted with a germinated *M. truncatula* seedling and inoculated with 2 mL of washed OD_{600} 0.05 WT CL150 culture or left uninoculated. Pots were placed in trays to prevent cross-contamination between inoculants and grown in a lighted growth chamber at 22°C and 150 µmol m^{-2} s^{-1} light on a 16 hr

light / 8 hr dark cycle. Pots were grown for 4 weeks with watering with 5 mL N-free rooting solution every 72 hours after the first week.

2.6.6 Image Acquisition

Microscope images were taken on a Leica M165 FC, with excitation light for fluorescence supplied by a Leica EL6000 fibre optic fluorescence light source. mCherry fluorescent protein was detected with ET mCherry filter (excitation: 560/40nm, emission: 630/74nm) and exposure time of 0.5s. Gain was set at 1x, saturation at 1.0 and gamma at 1. Leica Application Suite v4.12.0 was used to acquire images. Luminescence was measured using a Berthold NightOWL camera system with IndiGO v2.0.5.0 image analysis software. The minimum threshold for detected luminescence was set to 150 counts per second. All diagrams were made using BioRender scientific illustration software.

Chapter 3

Control of *nifA* for N_2 Fixation in *Azorhizobium caulinodans*

3.1 Overview

Engineering an associative diazotroph for more efficient fixed nitrogen delivery for cereal PGP is a promising strategy[165]. For example, the inoculation of maize with the diazotrophic strain *Pseudomonas protogens* Pf-5 X940 delivered fixed nitrogen to the cereal resulting in improved plant growth and nitrogen content[207,208]. The main hurdle of this strategy is overcoming the negative regulation of N_2 fixation by the cellular nitrogen and oxygen status, and the release of excess ammonia production to the target crop[46]. Furthermore, sub-optimal rhizosphere colonisation and non-specific colonisation of plant species could lead to ineffective nitrogen delivery, or growth promotion of non-target weed species[165]. Therefore, utilisation of host-derived signalling to control the expression of engineered N_2 fixation genes is essential to ensure specific induction of *nif* genes in the rhizosphere of target cereal species.

Analysis of plant root exudate shows plants produce unique secondary metabolites which can be harnessed to induce bacterial gene expression in the rhizosphere of target host plant species[54]. Previously, flavonoids and other secondary metabolites exudates from legumes have been used to control gene expression from biosensors in rhizobia colonising the rhizosphere of legumes[224]. Furthermore, biologically relevant signals from broad host plant species such as arabinose, salicylic acid, naringenin, and vanillic acid were used to regulate controllers for nitrogen fixation *in vitro* in root colonising bacteria such as *P. protogens* Pf-5[99]. Similarly, it was demonstrated that plant-microbe interactions may be engineered by inducing opine production in *Lotus corniculatus* by *Agrobacterium* mediated transformation, leading to enrichment of opine catabolizing bacteria in the plant rhizosphere[222]. However, many root exudates previously identified would make poor signals for engineered symbiosis due to the

lack of specificity between the host and cognate engineered bacterial symbiont, and signals such as opines induce pathogenicity in agrobacterium on plants[222].

Rhizopines, such as SI and 3-*O*-MSI are naturally produced by *Rhizobium* and *Sinorhizobium* strains hosted in legume nodules during symbiosis[229]. Cognate bacteria carrying *moc* catabolism genes for rhizopine can make use of this metabolite as a carbon and nitrogen source thus improving their nodulation competitiveness in the rhizosphere[225,231]. To harness rhizopines as a highly specific host-derived plant signal, the biosynthetic pathway for SI production constituting inositol dehydrogenase *idhA* and aminotransferase *mosB* were constitutively expressed from the roots of *H. vulgare* producing *RhiP* plant lines[227]. To demonstrate root exudation of rhizopine for bacterial gene induction, *R. leguminosarum* carrying a rhizopine biosensor pOPS0046 was tested on *RhiP* barley. The SI biosensor encoded *luxCDABE* under control of the *mocB* promotor, from the ATP-transporter substrate binding protein in the rhizopine catabolism locus, and its divergent transcription factor regulator *mocR*[231], and showed bioluminescence on the roots of engineered barley seedlings[227].

In this work, inositol-derived rhizopine has been used as a specific signal to control bacterial gene expression for control of *nif* induction. The engineering of the rhizopine trans-kingdom signal into the roots of cereals, such as barley, allows us to test the feasibility of secreted signalling between roots and engineered associative bacteria in the rhizosphere using a fluorescent biosensor based on the reporter circuit described above. Bacterial induction by rhizopine can then be developed to test control of associative nitrogen fixation *in vitro,* and in the rhizosphere induced by the host-dependent rhizopine signal. Control of nitrogen fixation coupled with NH_3 secretion would impart host specificity for targeted delivery of fixed nitrogen to engineered cereals and would prevent unintended interaction and growth-promotion of non-target plant species.

The synthetic rhizopine signal presents an excellent opportunity to demonstrate and develop synthetic control of associative nitrogen fixation in agricultural inoculants. Several rhizobia have been isolated from crop roots and rhizospheres which are cereal endophytes[62]. However, many are unable to effectively fix nitrogen under free living conditions due the oxygen sensitivity of the nitrogenase enzyme and the stringent regulation of *nif* genes required for fixation due to the metabolic burden it imparts on the cell[46]. The endophyte is *A. caulinodans* ORS571 is unusual in that as it is capable of fixing nitrogen in a symbiotic state in the nodules of its host plant *Sesbania rostrata,* and under free living conditions. Furthermore, *A. caulinodans* has been shown to be an endophytic coloniser of rice and wheat cereals, though with low levels of nitrogenase expression and subsequent nitrogen transfer[121,122,250]. *A. caulinodans* is a versatile nitrogen fixer as it is capable of synthesising a functional nitrogenase complex in the absence of a plant host due to a copy of the *nifV* gene which encodes the enzyme homocitrate synthase, homocitrate being a required co-factor for a functional MoFe nitrogenase cluster, as well as respiring with at least five terminal oxidases providing physiological versatility to fix nitrogen at different oxygen tensions[125,251]. *A. caulinodans* nitrogen fixation genes are controlled in a similar manner to many rhizobia, whereby *nif* gene expression is induced by the master transcriptional activator NifA in conjunction with the RpoN sigma factor[84,130]. In turn, cellular oxygen concentration, via FixLJK activity, and nitrogen concentration, controlled by the two component regulatory systems NtrBC and NtrYX, control *nifA* expression and activity. The convergence of cellular signalling on NifA make it a prime engineering target to establish control of N_2 fixation.

It has previously been demonstrated that tight regulation of free living nitrogen fixation can be achieved by placing *nifA-rpoN* expression under control of synthetic or root-exudate derived chemicals for induction of downstream *nif* and *fix* genes required for a functional nitrogenase[99]. Synthetic transcriptional control of *nifA-rpoN* in a Δ*nifA* background strain

removes the native transcriptional repression of the *nifA* activator by cellular N and O_2 status. However, further poorly understood cellular regulators repressed NifA by post-translational mechanisms, although these were partially overcome by encoding a $nifA_{L94Q/D95Q}$ variant carrying a mutation in its GAF regulatory domain[99]. It is important that a viable agricultural inoculant has stringent control over induction of nitrogen fixation, as constitutive activity imparts a costly fitness burden on bacterial cells resulting in poor nitrogen production and population decline[207].

Here, we present a strategy to use the orthogonal and biologically relevant synthetic rhizopine signal to demonstrate control of *in vitro* nitrogen fixation via *nifA* expression in *A. caulinodans*. First, rhizopine biosensors were tested and tuned in *A. caulinodans* to optimise expression of *nifA* in response to SI. An assay was developed to show specific induction of *nif* genes in response to the exogenous rhizopine signal under microaerobic conditions via a fluorescent report construct driven by a nitrogenase specific promoter. Induction by rhizopine was followed by an *in vitro* assay to demonstrate partially ammonium insensitive free living nitrogen fixation achieved by rhizopine induction of a controller expressing a mutant *nifA*. Nitrogen secretion to the media was assayed in this strain under microaerobic conditions but none was detected.

3.2 Rhizopine Biosensors in *A. caulinodans*

Initial work in this lab to develop a rhizopine dependent biosensor took genetic components from the rhizopine catabolising strain *S. meliloti* L5-30 previously characterised in this lab [227,248]. The transcription factor MocR which drives rhizopine dependent expression from the P*mocB* promoter, upstream of *mocBA* catabolism genes, were encoded on the PMQ131PAR-derived broad host range receiver plasmid pOPS0759 with P*mocB* driving *gfp* expression (OPS1077) (*Figure 3.1*). Initial measurements of rhizopine induction of

P*mocB::gfp* expression were poor due to 1 mM of rhizopine only producing a two-fold induction over background fluorescence[201]. To increase rhizopine sensitivity, a second receiver plasmid was constructed by Dr Timothy Haskett in this laboratory by co-expressing a solute binding protein MocB, also from *S. meliloti* L5-30, from a low expression Anderson promotor P*J23115* producing plasmid pOPS1495 (OPS2202). However, this failed to increase the sensitivity or dynamic range of rhizopine dependent *gfp* expression in *A. caulinodans*[201]. We theorised that transport of rhizopine into the cell was poor due to lack of transmembrane transporter components. Whilst no specific transmembrane components for rhizopine transport in bacteria have been identified, an ATP-binding cassette transporter IntABC was identified in *Pisum sativum* bacterial symbiont *R. leguminosarum* bv3841, which transports the structurally similar molecule *myo*-inositol. Structurally, *scyllo*-inositol differs from *myo*-inositol due to the substitution of one hydroxyl group for an amine group. Similarly, the amino acid sequence of the IntA solute binding protein was aligned to MocB from *S. meliloti* L5-30 and was found to be 78.32% identical[201]. Given the close homology of the solute binding proteins and their substrates, we tested whether the transmembrane components IntBC could interact with MocB to transport rhizopine. To test this, *R. leguminosarum* carrying the pOPS0761 receiver plasmid was grown on media containing *myo*-inositol as a carbon source to induce *intABC* transporter expression resulting in six-fold GFP fluorescence compared to the control[201].

A second rhizopine receiver plasmid was constructed by amplifying *mocR*-P*mocB* with primers oxp2115/oxp1868; and the *R. leguminosarum intBC* genes were amplified using primers oxp1431/1432 and cloned downstream of P*J23115*::*mocB* to form plasmid pOPS0601. From this plasmid, P*J23115*::*mocB-intBC* was amplified with primers oxp2114/1856; and the *gfp*-DT15 locus was amplified from pOGG063 with primers oxp1869/1336. *Gfp*-DT15 was then assembled downstream of P*J23115*::*mocB-intBC* by yeast recombination into the

pMQ131PAR shuttle vector digested with BamH1 to form plasmid pOPS0889 (OPS1204) (*Figure 3.1*).

To test whether inclusion of *intBC* transporters in the rhizopine biosensor pOPS0889 improved sensitivity and inducible expression in *A. caulinodans,* the strains carrying pOPS0889 and pOPS1495 (OPS1204 and OPS2202 respectively) were grown overnight in TY media before centrifugation to pellet cultures and resuspended at OD_{600} 0.3 in UMS minimal media supplemented with increasing concentrations of rhizopine and incubated for 18 hours before measurement of GFP fluorescence in a microplate reader. The expression of P*mocB::gfp* in *A. caulinodans* carrying the pOPS0889 biosensor with *intBC* genes showed increased expression across all concentrations of SI tested, with maximum induction seen as low as 10 µM SI. In comparison, the pOPS1495 biosensor only expressing the *mocB* SBP required 1 mM of SI to induce expression to double the basal level (*Figure 3.1*). Inclusion of *intBC* transporter genes thereby increases the sensitivity and relative gene expression in response to rhizopine.

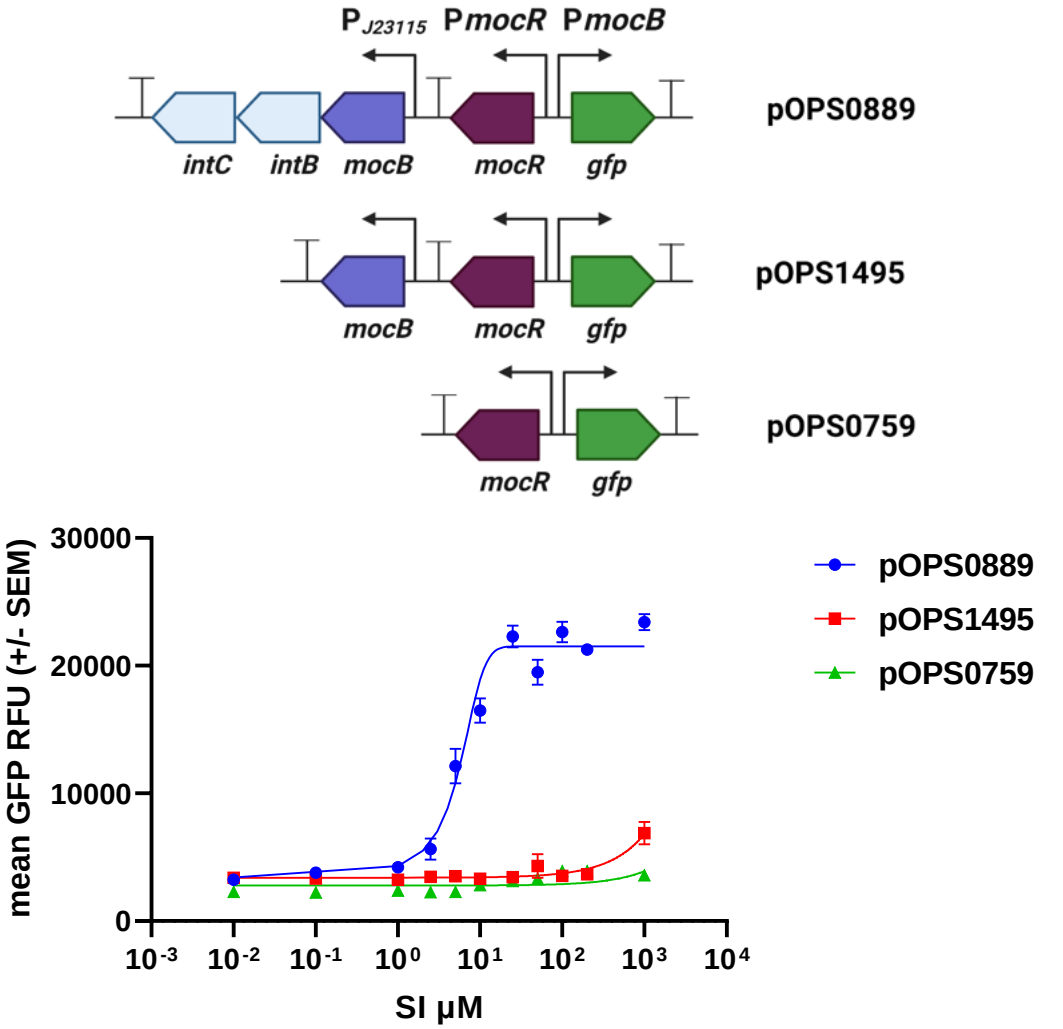


Figure 3.1 Tuning rhizopine biosensor expression in *A. caulinodans*. Design of three pBBR1 plasmid biosensors expressing *gfp* from P*mocB* which is induced by MocR in response to SI. Plasmid pOPS1495 includes expression of the MocB solute binding protein from the weak Anderson promotor P*J23115*, whilst pOPS0889 also encodes *intBC* transport genes (top). Dose response curve for *gfp* induction in *A. caulinodans* carrying pOPS0759, pOPS1495, or pOPS0889. Bacteria were induced for 24 hours prior to measurement. Error bars represent one SEM (n = 3).

3.3 Construction of SI Inducible *nifA-rpoN A. caulinodans* Strains

The second generation rhizopine biosensor pOPS0889 demonstrated increased sensitivity and dynamic range to exogenous rhizopine and can therefore be used as a backbone to develop SI dependent control of nitrogen fixation. Expression of the central *nif* regulatory transcription factor *nifA* and its associated sigma factor *rpoN* can be placed under control of

P*mocB* expression, and the resulting plasmid conjugated into a Δ*nifA A. caulinodans* strain, to render control of N_2 fixation in the strain by application of rhizopine (*Figure 3.2*). The circuit design has the attached benefit of circumventing native transcriptional repression of *nifA* by the nitrogen regulated NtrBC and oxygen regulated FixLJ cascades. Therefore, the *nifA* and *rpoN* cassette was PCR amplified from pMR124 by primers oxp2319/1438, with a strong RBS encoded on the forward primer, and HiFi cloned into pOPS0889 digested with BamH1 to produced SI inducible NifA controller pOPS1122 (OPS1626).

Additionally, A *nifA* mutant in the related alphaprotebacteria, *R. capsulatus,* was previously identified which relieves ammonium negative-feedback repression[139]. The equivalent mutations were identified in *A. caulinodans* resulting in the inclusion of two amino acid substitutions L94Q/D95Q in the GAF regulatory domain of NifA in an inducible controller plasmid which partially overcame post-translational ammonium repression of the transcription factor[99]. Therefore, a SI inducible NifA controller was constructed via amplification of $nifA_{L94Q/D95Q}$ with primers oxp2319/oxp4009 from plasmid pOGG290 and *rpoN* was amplified with oxp4010/1438 from *A. caulinodans* with both fragments assembled by HiFi cloning into BamH1 digested pOPS0889 to produced SI controller plasmid pOPS1476 (OPS2624) (*Figure 3.2*). The plasmid sequence was confirmed by Sanger sequencing with primers oxp3346/1283.

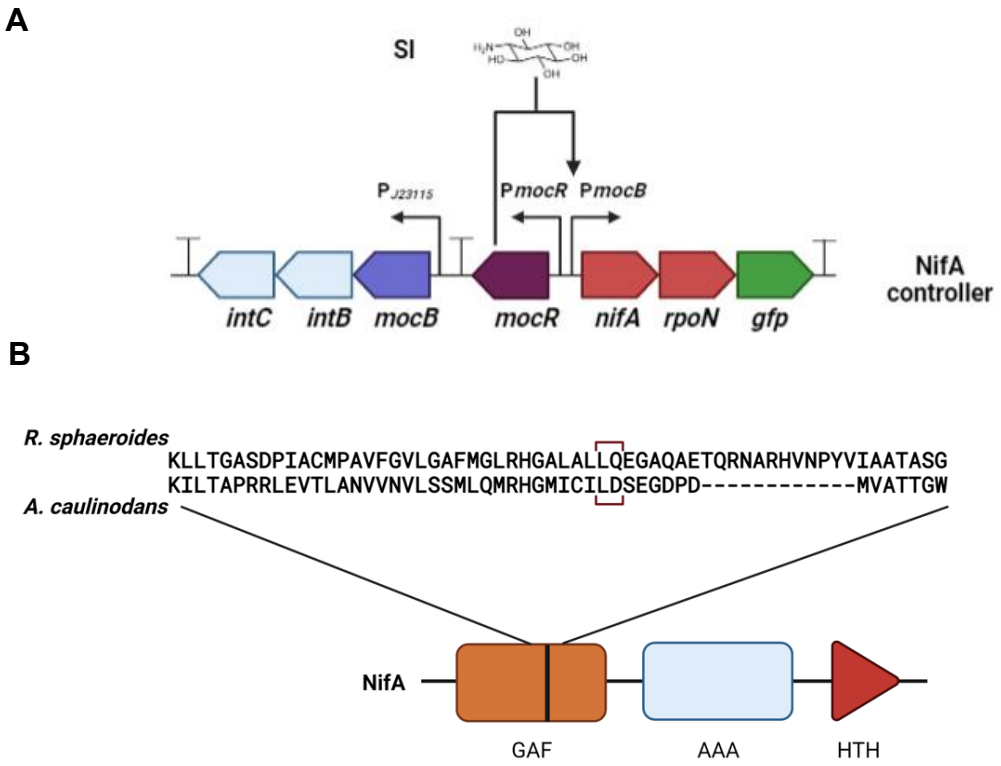


Figure 3.2 A Rhizopine inducible NifA controller constructed by placing *nifA-rpoN* under control of *PmocB* in rhizopine biosensor pOPS0889. **B** The domain structure of *A. caulinodans* NifA, with GAF regulatory domain shown in orange, AAA^+ ATPase domains are shown in light blue, and the helix-turn-helix DNA binding domain shown in red. The MUSCLE alignment of *R. sphaeroides* NifA to *A. caulinodans* NifA is shown above with the L94/D95 region for mutation to impart nitrogen insensitivity shown highlighted in red.

3.4 Testing Nitrogenase Induction by SI Inducible *nifA*

To initially measure nitrogenase gene activation by NifA controllers induced by exogenous SI, a reporter for *nif* gene expression was developed. *A. caulinodans* nitrogenase induction in response to rhizopine could be assayed using the *nifH1* promoter from the dinitrogenase reductase gene *nifH*, required for a functional nitrogenase enzyme under free living conditions. A transcriptional block of P*nifH*::mCherry-Tpharma previously constructed in this lab via golden gate assembly[201] was amplified from pOPS1214 with primers

oxp2960/2961 and assembled with HiFi master mix into the broad host range vector pRK415 digested with EcoR1 to produce plasmid pOPS1218.

Initially an assay was developed to check induction of the P*nifH* reporter construct under microaerobic conditions. The P*nifH::mCherry* reporter was conjugated into WT *A. caulinodans* and *A. caulinodans ΔnifA* markerless deletion mutant previously constructed in this laboratory. Due to difficulty with growing *A. caulinodans* under microaerobic conditions in 96 well plates, the P*nifH* induction assay was performed in cultures grown in McCartney vials in UMS supplemented with 20 mM succinate at a starting OD of 0.3, with incubation at 3% oxygen in a microaerobic cabinet for 4 hours, before sealing the vials and growing the cultures for 18 hours at 37°C with shaking. Endpoint measurements for OD and mCherry induction were measured in a 96 well plate reader following incubation. WT *A. caulinodans* carrying P*nifH* reporter pOPS1218 showed 10^3-fold induction of mCherry fluorescence under microaerobic conditions whilst no induction of the reporter was seen in the *A. caulinodans ΔnifA* strain, confirming specific *PnifH::mCherry* activation by NifA under free living nitrogen fixing conditions (*Figure 3.3*). However, in the WT *A. caulinodans* strain, the induction of P*nifH* was abolished by the presence of 10 mM NH_4Cl in the assay growth medium, demonstrating the native nitrogen repression of *nifA* (*Figure 3.3*)[46,130].

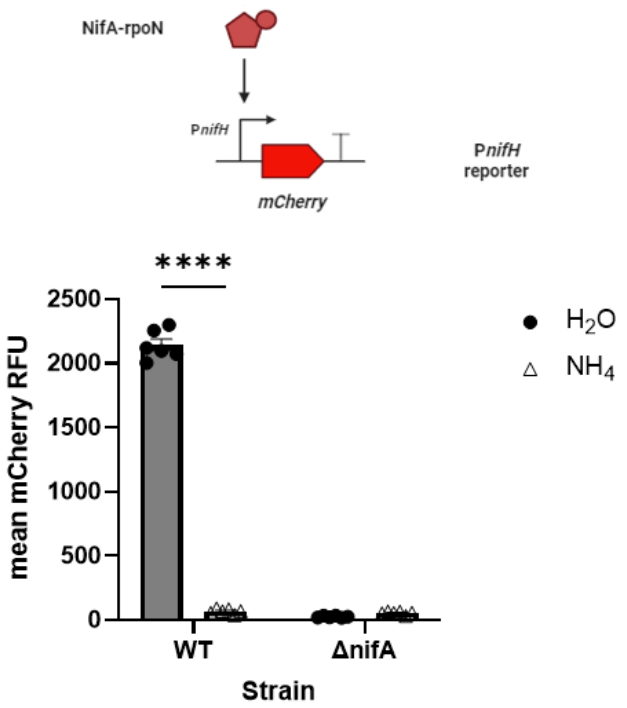


Figure 3.3 Schematic of NifA-RpoN induction of P*nifH::mCherry* reporter plasmid pOPS1218 in *A. caulinodans* (top). Relative fluorescence of P*nifH::mCherry* reporter in WT and *ΔnifA* strains of *A. caulinodans* grown in minimal media supplemented with 20 mM succinate with 3% O_2 headspace (bottom). Induction was measured after 18 hours incubation. (Error bars represent one SEM, n = 6, Independent two-tailed Student's t tests were used to compare means, **** $p \leq 0.001$).

To demonstrate SI dependent induction of *nif* genes under free living conditions, both SI NifA controller plasmids, pOPS1122 and pOPS1476, were conjugated into *A. caulinodans ΔnifA* alongside the P*nifH* reporter pOPS1218 by tri-parental mating (producing OPS2622 and OPS2620 respectively). Both WT and Δ*nifA* strains carrying pOPS1218 in the absence of SI plasmid controllers were also included as controls. An induction assay under free living microaerobic conditions was performed with these strains using the same method as previously discussed. However, further media compositions were tested to determine the effect of ammonium regulation on P*nifH* induction with each SI NifA controller. Three biological replicates of each strain were tested in UMS supplemented with 20 mM succinate, UMS with

20 mM succinate and 10 µM SI, and UMS with 20 mM succinate, 10 mM NH_4Cl, and 10 µM SI.

Activation of the *nifH* promoter by native NifA was seen in the WT with no significant difference in expression caused by the addition of 10 µM SI, and as previously shown, P*nifH::mCherry* induction was severely inhibited by the addition of ammonium to the assay media (*Figure 3.4*). In *A. caulinodans* Δ*nifA* strains, no mCherry expression was seen in the absence of SI NifA controllers pOPS1122 and pOPS1476 due to lack of a functional NifA transcription factor. Basal levels of P*nifH::mCherry* expression were detectable with both SI NifA controller strains in the absence of rhizopine, though the leaky induction was significantly lower than the induced state ($p \leq 0.001$) (*Figure 3.4*). Addition of 10 µM SI with either of the NifA controllers resulted in greater induction of P*nifH* than seen with WT *A. caulinodans* (*Figure 3.4*). Expression of *nifA-rpoN* and $nifA_{L94Q/D95Q}$-*rpoN* by SI, from controller pOPS1122 and pOPS1476 in the presence of 10 mM NH_4Cl resulted in partial induction of P*nifH,* though this was only statistically significant from the uninduced state with SI controller $nifA_{L94Q/D95Q}$-*rpoN* (pOPS1476, $p \leq 0.05$). Expression by rhizopine of the GAF domain NifA mutant (pOPS1476) gave greater induction from P*nifH* under high nitrogen conditions than the controller expressing a native *nifA* gene (pOPS1122), at ~50% and ~15% of WT *A. caulinodans* respectively. Demonstrating that SI can control *nifA* transcription factor expression and therefore induction of *nif* genes under microaerobic conditions, and that mutation of two GAF domain residues partially alleviates ammonium repression of NifA activity.

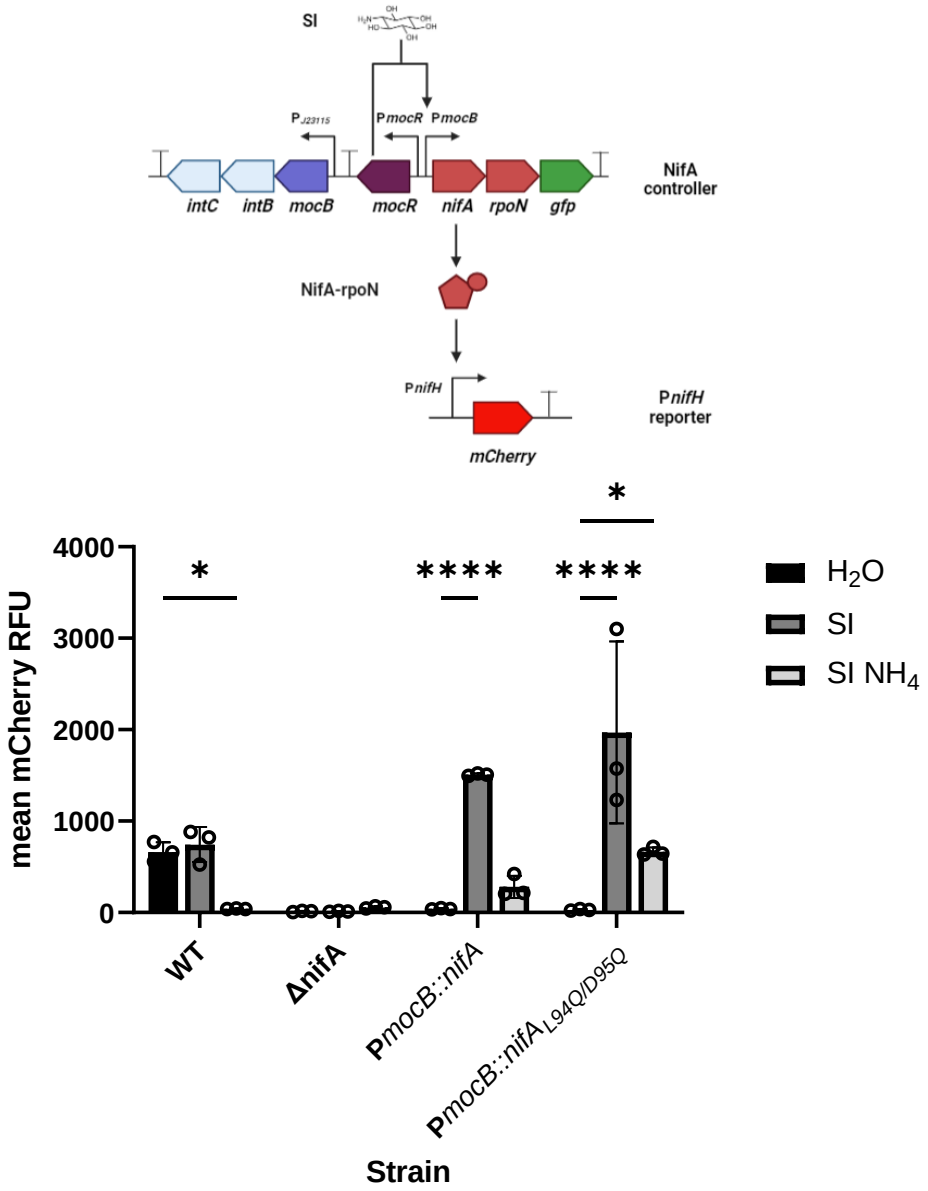


Figure 3.4 Schematic of SI induction of *nifA-rpoN* from rhizopine controller plasmids pOPS1122 and pOPS1476 encoding P*mocB::nifA-rpoN* and $nifA_{L94Q/D95Q}$-*rpoN* respectively, for measurement of P*nifH* promoter activity from pOPS1218 reporter (top). P*nifH* promoter activity RFU, defined as mCherry fluorescence/OD_{600} was measured in populations of *A. caulinodans* WT and *ΔnifA* strains carrying rhizopine controller plasmids and the reporter pOPS1218 grown in N_2-fixing conditions for 18 hours (N-free UMS supplemented with 20 mM succinate with a starting headspace of 3% O_2). SI and NH_4Cl was added to a final concentration of 10 µM and 10 mM respectively. Error bars represent one SEM, n = 3. Two-way ANOVA followed by Tukey's post-hoc analysis was performed to compare means, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.001$.

3.5 Testing Nitrogen Fixation by SI Dependent NifA Controllers

To determine whether the NifA controllers developed could establish rhizopine dependent control of nitrogen fixation in *A. caulinodans*, acetylene reduction assays (ARAs) were used to assess nitrogenase activity under microaerobic conditions. ARAs can be used as an indirect method of measuring the rate of nitrogen fixation using the ability of the bacterial nitrogenase enzyme to reduce the triple bond of acetylene, followed by measuring the ratio of the ethylene product to acetylene present in the assay headspace atmosphere using gas chromatography. A bicinchoninic acid assay (BCA) was then performed to measure the whole-cell protein content of the bacterial culture to standardise the rates of ethylene production between assay replicates.

Initially, free living microaerobic conditions were tested to develop reliable conditions for ARAs in *A. caulinodans.* As *A. caulinodans* is an obligate aerobe but requires low cellular oxygen concentrations for optimal nitrogenase activity, Ryu *et al*, 2019, tested the tolerance of both WT and inducible *nifA* strains of ORS571 to oxygen as a function of the headspace oxygen concentration present in the assay. Their results showed that nitrogenase activity increased with decreasing assay oxygen percentage for either strain, with a maximum fixation rate at 0.5-1% headspace O_2[99]. We replicated these results using our experimental setup with WT *A. caulinodans* to confirm fixation under microaerobic conditions. Overnight cultures of *A. caulinodans* were centrifuged and washed thrice with PBS to remove access nitrogen before resuspension in 2 mL UMS at OD_{600} of 0.3, followed by initiation of the assay by incubation at 3% or 1% oxygen for 2 hours. As *A. caulinodans* is a fast-growing species, the vials of culture were sealed and incubated with shaking for a shorter time, 9 hours, and at lower temperature, 30°C, than previous experiments to ensure logarithmic growth over the course of the assay. To measure fixation rates after incubation, 10% of the vial headspace volume was replaced with acetylene and 1 mL extracted for GC-MS analysis after 4 hours. Additionally,

the oxygen concentration of sealed cultures was measured during the incubation using OxySense non-invasive measurements.

Over the course of the assay, cultures induced at 3% O_2 recorded a final mean oxygen concentration of 1%, whilst cultures initiated at 1% O_2 ended at 0.4% after 9 hours incubation (*Figure* 3.5). No difference in growth was seen in cultures at either O_2 concentrations, with culture populations approximately doubling over the course of the assay (*Figure* 3.5). The reduction in oxygen concentration shows that bacterial cultures were actively respiring during division over the incubation period, and both cultures resulted in an optimal oxygen concentration before initiation of the ARA. Furthermore, no significant difference was detected in the resulting rates of N_2 fixation between cultures at different starting O_2 concentrations (*Figure* 3.5). No acetylene reduction was seen in *A. caulinodans* Δ*nifA* strains, confirming no fixation is present in strains lacking a functional copy of the *nif* transcriptional regulator.

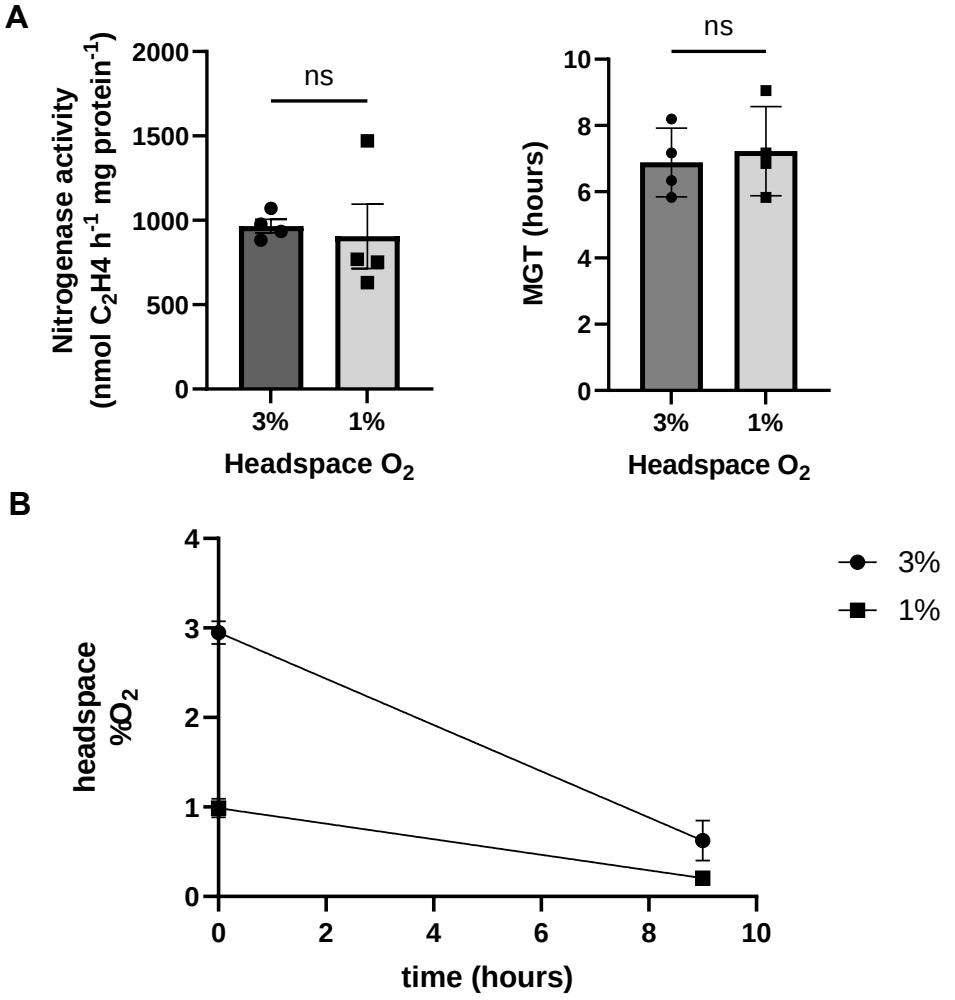


Figure 3.5 *A. caulinodans* nitrogenase activity under different microaerobic conditions **A)** Nitrogenase activity was assessed by acetylene reduction assay on WT *A. caulinodans* cultures at different starting headspace O_2 concentrations, cultures were incubated for 9 hours with shaking at 30°C in minimal media (5 mL cultures in N-free UMS supplemented with 20 mM succinate), nitrogenase activity was assessed by addition of 10% acetylene to the headspace and measured over 4 hours incubation. Error bars represent one SEM, n = 4. **B)** OD_{600} measurements were taken from WT cultures at the start and end of incubation and used to calculate mean generation time (MGT), Error bars represent one SEM, n = 4. Student's T-test was used to compare means **C)** headspace oxygen concentration of WT cultures was measured the start and end of 9-hour incubation Error bars represent one SEM, n = 4, ns = no significant difference.

Using the same free living diazotrophic assay method, rates of nitrogen fixation were measured for both rhizopine inducible controllers P*mocB::nifA-rpoN* (pOPS1122) and P*mocB::nifAL94Q$_{/D95Q}$-rpoN* (pOPS1476) with and without 10 μM SI and compared to the rates of WT *A. caulinodans* and Δ*nifA* strain under different nitrogen conditions. The number of biological replicates was increased from three to eight to reduce the variability seen in nitrogenase activity in cultures of WT *A. caulinodans* (*Figure 3.5*). No nitrogenase activity was seen in either NifA controller strain in the absence of SI, whilst addition of 10 μM SI resulted in comparable rates of nitrogen fixation to the WT strain (*Figure 3.6*). Again, the WT strain showed repression of nitrogenase activity in media supplemented with 10 mM NH_4Cl, whist rhizopine induction of *nifA* was able to partially overcome nitrogen repression, with the GAF domain mutant pOPS1476 showing greater activity at ~50% of WT levels under high nitrogen conditions (*Figure 3.6*). This nitrogenase reduction assay demonstrates tight binary control of free living nitrogen fixation by NifA controller strains in an SI dependent manner.

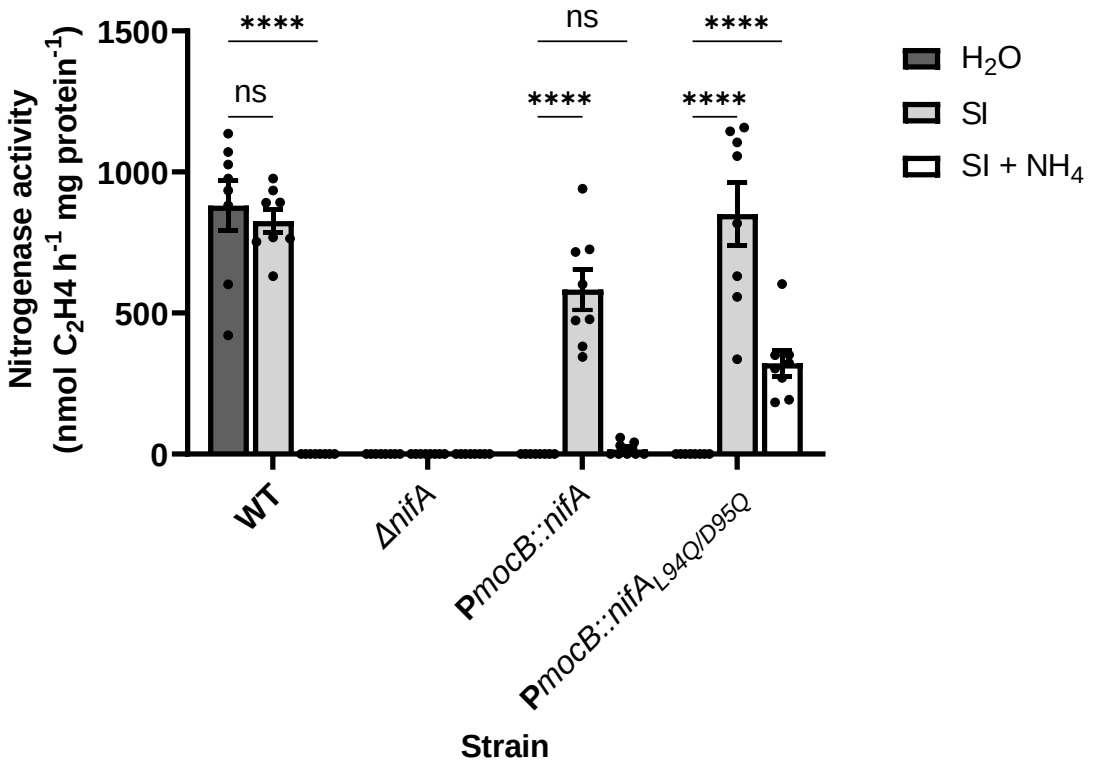


Figure 3.6 Nitrogenase activity was assessed in cultures of *A. caulinodans* WT and *ΔnifA* strains carrying rhizopine controller plasmids P*mocB::nifA-rpoN* (pOPS1122) and P*mocB::nifAL94Q$_{D95Q}$-rpoN* (pOPS1476) grown in N_2-fixing conditions for 9 hours (N-free UMS supplemented with 20 mM succinate with a starting headspace of 3% O_2). Nitrogenase activity was assessed by addition of 10% acetylene to the headspace and measured over 4 hours incubation. SI and NH_4Cl was added to a final concentration of 10 μM and 10 mM respectively where indicated. Error bars represent one SEM, n = 8. Two-way ANOVA followed by Tukey's post-hoc analysis was performed to compare means, ns $p > 0.05$, **** $p \leq 0.001$.

3.6 Testing NH_3 Secretion of SI Controller Strains

Whilst we have shown control of nitrogen fixation *in vitro* in response to a synthetic signal, this does not guarantee fixed nitrogen release by the engineered bacterium which would be required of an effective agricultural inoculant. Cellular NH_3 production and assimilation are tightly coupled by regulatory circuits which channel fixed nitrogen into biomass production to prevent loss by diffusion from the cell. The cellular nitrogen status is primarily sensed by PII signal transduction proteins which are subject to post-translational modification by the bi-

functional uridyltransferase GlnD, which can add or remove uridylyl groups from PII depending on the nitrogen status. PII protein modification affects the regulation of downstream targets of nitrogen metabolism, such as glutamine synthetase (GS) enzyme[129,136,212].

Engineering of excess ammonia excretion has been achieved in associative diazotrophs by disrupting key regulators of nitrogen fixation and assimilation, though these strategies are not universal due to nuances in regulatory cascades between bacteria[46]. In the gamma-proteobacterium *A. vinelandii*, the *nifL* gene which combines O_2 and N_2 sensing, acts to repress nitrogenase through binding and inhibiting NifA. *A. vinelandii* can be engineered to release NH_3 by deleting *nifL* or mutating the GAF domain of NifA to prevent binding[86,149,153,215]. Whilst chemical induction of a mutated *glnA* in *A. vinelandii* and of unidirectional adenylyltransferase *glnE* in *A. brasilense* have resulted in ammonia secretion phenotypes[213,252,253].

To test whether nitrogen fixation driven by the partially nitrogen insensitive P*mocB::nifAL94Q*$_{D95Q}$*-rpoN* releases fixed nitrogen as NH_3 when induced by SI under microaerobic conditions, we performed a spectrophotometric assay to quantify ammonia as indophenol. *A. caulinodans* cultures were grown for 24 hours in N-free UMS with 10 µM rhizopine under microaerobic conditions. Culture supernatants were collected by centrifugation and measured for the presence of ammonia by reaction with indophenol. No ammonia was detected in the supernatant of either WT *A. caulinodans* or SI induced P*mocB::nifA*$_{L94Q/D95Q}$*-rpoN* (pOPS1476) strains when compared to NH_3 standards after incubation under microaerobic conditions ($p \leq 0.001$, *Figure 3.7*). Therefore, fixed nitrogen is still being assimilated by the bacterium rather than secreted into the media, despite partial alleviation of ammonia repression on nitrogen fixation via the encoded *nifA* mutant.

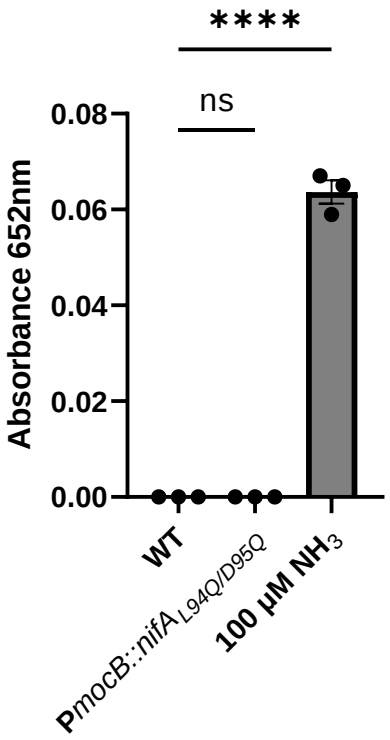


Figure 3.7 Spectrophotometric determination of NH_3 secretion in the supernatant of cultures of *A. caulinodans* WT and *ΔnifA* strain carrying rhizopine controller plasmids P*mocB::nifAL94Q*$_{/D95Q}$-*rpoN* (pOPS1476) induced by 10 µM SI, grown in N_2-fixing conditions for 18 hours (N-free UMS supplemented with 20 mM succinate with a starting headspace of 3% O_2), A 100 µM NH_3 solution was included as a positive control. Error bars represent one SEM, n = 3. Independent two-tailed Student's T-test was performed to compare means relative to the WT, ns $p > 0.05$, **** $p \leq 0.001$.

3.7 Deletion of PII Genes in *A. caulinodans*

Many proteobacteria possess multiple PII homologues, with *A. caulinodans* possessing two PII proteins GlnB and GlnK. It has previously been reported that disruption of *A. caulinodans glnB* and *glnK* by transposon mutagenesis resulted in ammonium secretion under free living nitrogen fixation due to high levels of GS adenylylation and nitrogenase depression[129]. To replicate these results in our engineered *A. caulinodans* strain we attempted to construct a double markerless deletion of *glnB* and *glnK* using the pk19mobsacB suicide vector and selecting for homologous recombination at the chromosomal region of interest[254].

First, deletion of *glnB* was tested using plasmid pOPS1691, constructed by amplifying flanking genomic regions of *glnB* from *A. caulinodans* genomic DNA with oxp4186/4187 and HiFi cloning into pK19mobSacB digested by SmaI. The pOPS1691 plasmid was mobilised into *A. caulinodans* by triparental mating and a single-crossover integration event selected by plating on TY agar with 100µg mL^{-1} Kanamycin. Successful single-crossover mutants were subsequently grown in non-selective media to stationary phase and plated on TY agar containing 10% v/v sucrose to select for double-crossover deletion of the target gene. We were successful in producing a *glnB* knockout strain of *Azorhizobium* (OPS2222), which was confirmed by streaking colonies on media plus and minus Kanamycin and screening sensitive colonies by PCR and Sanger sequencing of the *glnB* genomic region. To produce a deletion of the second PII gene *glnK,* a second pK19mobSacB vector was constructed by amplification of *glnK* genomic regions with oxp4182-4185 and cloning the fragment alongside the pHP45 ΩSpec cassette into the EcoRI and HindIII digested vector to produce *ΔglnK*::Ω-Spec replacement plasmid pOPS1564. The resulting *glnK* deletion vector was mobilised into *A. caulinodans* Δ*glnB* strains and selected for single and double crossover events using the same protocol previously described. We were unable to produce a double mutant of Δ*glnB glnK* by introducing the second *glnK* deletion vector on multiple attempts, even when selecting for mutants on media supplemented with glutamine as a replacement nitrogen source. The failure to produce a double mutant suggests that the resulting knockout is lethal due to the strain being growth defective. Single knockout strains of *Ac ΔglnB* or *ΔglnK*::Ω-Spec (OPS2379) were obtained but did not secrete NH_3 to the growth media when assayed with the indophenol spectrophotometric method, suggesting one copy is sufficient to regulate GS activity[126].

3.8 Discussion

In this chapter, we have built and analysed the feasibility of rhizopine-dependent controllers for *nif* gene expression in *Azorhizobium*. We tuned the expression of the previously

reported biosensor from work in this group[227] by inclusion of rhizopine uptake genes from *R. leguminosarum*. The improved rhizopine signalling circuitry was used to demonstrate control of *in vitro* nitrogen fixation by driving expression of the transcriptional regulators *nifA-rpoN*. We first showed control of NifA was capable of inducing expression of downstream *nif* genes in a rhizopine dependent manner. Induction was followed by demonstration of *in vitro* nitrogen fixation using the developed controller strains which were partially insensitive to the presence of fixed nitrogen due to transcriptional control and mutations in the GAF regulatory domain. *Azorhizobium* NifA controller strains were capable of fixing atmospheric nitrogen (*Figure 3.6*) but were unable to release excess ammonium to the environment (*Figure 3.7*), attempts to engineer ammonium release by markerless deletion of PII regulatory proteins failed, as a viable double-deletion strain could not be generated. The rhizopine dependent signalling circuit in *A. caulinodans* provides a platform to engineer plant host control of associative nitrogen fixation for cereals.

It was previously reported that a rhizopine biosensor (pOPS0761), made by cloning the *mocB* promoter and its divergent regulator *mocR* from *S. meliloti* into a reporter vector pMQ131par, could respond to rhizopine in the rhizosphere of T_0 barley seedlings[227]. In order to generate robust control of nitrogen fixation in engineered strains we improved the sensitivity and dynamic range of the rhizopine biosensor by inclusion of the *S. meliloti* L5-30 solute binding protein MocB, also found in the *moc* catabolism operon, as well as the inositol ABC-type transporter IntBC from *R. leguminosarum Rlv*3841. Inclusion of the *mocB* gene in the rhizopine biosensor pOPS1495 was enough to improve the sensitivity over the initial *mocR-PmocB::gfp* biosensor pOPS0759, however it still required 1 mM SI to induced *gfp* expression above the baseline level and only elicited a two-fold change in induction (*Figure 3.1*). Induction of pOPS1495 suggests that diffusion of rhizopine into the cell was not enough to produce a robust response to the signal, therefore *intBC* transporters were also encoded under

the same low expression promotor in the biosensor pOPS0889. Expression of *gfp* using pOPS0889 was improved across all concentrations of SI tested with induction seen at 1µM and maximal induction at 10 µM, with sensitivity of the biosensor response to SI improved by a factor of 10^3 over the previous biosensor (*Figure 3.1*). To demonstrate the IntBC transporters functioned as an SI uptake system, *Rlv3841* carrying a *mocR*-P*mocB::luxCDABE* was grown on media containing myo-inositol to induce expression of the chromosomal *intABC* genes, which led to increased P*mocB* promotor activity. Induction of P*mocB* was replicated in an *Rlv3841 intA*::mini-Tn5 mutant strain, where *intABC* expression was abolished, carrying the P*mocB::luxCDABE* biosensor. P*mocB* activity could be restored in this strain in response to rhizopine by complementation with a constitutively expressed P*lac::intBC* plasmid[248], demonstrating that *intBC* genes are required for a sensitive SI dependent transcription from P*mocB*.

Further analysis of induction in *A. caulinodans* strains carrying the pOPS0889 rhizopine biosensor with the hybrid SI transport system showed that despite the increase in SI-perception, only a small subset of cells responded to the SI signal. Flow cytometry was performed on mCherry labelled strains carrying the SI biosensor pOPS0889 with only ~36% of cells exhibiting *gfp* expression above the threshold for induction, defined as mean 99th percentile of noninduced cells[201]. A similar analysis was performed on the same SI biosensor strain isolated from the roots of T2 *RhiP* barley secreting rhizopine into the rhizosphere. SI dependent *gfp* expression was only found in only 5% of the bacterial cells colonising T2 barley[201]. It is unlikely that plasmid loss caused a reduction in rhizopine perception in both these experiments as pMQ131 plasmids encode PAR genes for plasmid maintenance, and cells recovered from both *in vitro* and *in planta* induction were positive for kanamycin resistance harboured on the SI biosensor[201]. It is possible that constitutive transporter expression could lead to over-accumulation of carbon substrates by active transport leading to cellular toxicity

resulting in a growth defect and possible silencing of rhizopine perception by plasmid mutation. A silencing analysis was performed on *A. caulinodans* pOPS0889 recovered from the roots of *RhiP* barley by re-culturing the bacterial population in minimal media and re-inducing the cells with 10 μM SI for 48 hours. Subsequent flow-cytometry analysis showed that only 3% of recovered cells from the root carrying the pOPS0889 biosensor were able to respond to rhizopine upon re-induction, versus 65% of cells recovered from the washed assay media fraction[248]. The reduction in sensitivity suggests rhizopine toxicity is a factor for reduced SI perception in biosensor strains, resulting in reduced stability of gene expression *in vitro* and in the rhizoplane of *RhiP* plants. The robustness of SI-dependent expression could be improved for further engineered biosensor circuits by reducing the plasmid copy number, or the expression levels of *intBC* transporters which accumulate SI.

To generate rhizopine dependent control of nitrogen fixation in *A. caulinodans* the SI biosensor pOPS0889 was used as a backbone to generate *nifA-rpoN* and *nifA*$_{l94Q/D95Q}$*-rpoN* controller plasmids pOPS1122 and pOPS1476. These controllers take advantage of the native hierarchical regulation of *nif/fix* genes required for fixation which converges on the NifA transcription factor[130,150,214]. These controllers were mobilised into the Δ*nifA A. caulinodans* strain and their functionality tested. Firstly, their ability to induce expression of nitrogenase structural genes in response to SI was tested using the P*nifH* reporter plasmid pOPS1218 under diazotrophic conditions (*Figure 3.3*). Basal levels of P*nifH::mCherry* were detected in both NifA controllers in the absence of SI, probably due to the leaky expression of P*mocB,* which can be observed in the increased baseline of pOPS0889 induction curves (*Figure 3.1*). Induction of both NifA controllers with SI resulted in higher P*nifH* expression relative to WT strain induction, demonstrating strong expression of *nifA* in response to rhizopine (*Figure 3.4*).

As previously reported[99], removal of native transcriptional regulation and expression by exogenous inducer of *nifA*$_{l94Q/D95Q}$*-rpoN* results in partial P*nifH* expression in the presence

of ammonium in the growth media, whilst P*nifH* induction by NifA-RpoN under high N conditions was not significantly different from the H_2O control ($p = 0.097$). As expected, the *nifA*$_{l94Q/D95Q}$-*rpoN* cassette gave higher relative partial activity under high nitrogen conditions due to the GAF domain mutation, though still exhibited a 50% reduction compared to induction by SI in the absence of ammonium (*Figure 3.4*). Partial activity suggests post-translational regulation of NifA or nitrogenase in response to the cellular nitrogen status by a currently uncharacterised mechanism, demonstrating the multilayered regulation of N_2 fixation to ensure free living fixation is only carried out under nitrogen starvation conditions.

An optimised assay for measuring nitrogen fixation under microaerobic conditions in response to *nifA* induction was also developed, based on acetylene reduction as a proxy for nitrogenase activity[255,256] (*Figure 3.5*). Oxysense and OD_{600} measurements confirmed cultures were actively dividing under microaerobic assay conditions, though at a slower rate than reported doubling times for WT cultures under normal atmospheric conditions[126]. This reduction is likely due to the nitrogen free minimal media used in the assay and the induction of nitrogen fixation mechanisms under microaerobic conditions imposing a metabolic burden on the cells[46]. Acetylene reduction assays performed under 3% oxygen conditions in the absence of nitrogen showed rates of nitrogen fixation in rhizopine induced controller strains comparable to that of WT strains. No fixation was seen in the absence of rhizopine, demonstrating tight-binary control of nitrogenase activity by NifA controller plasmids, an improvement on previously reported inducible NifA controllers[99]. Under high nitrogen conditions, only the *nifA*$_{L94Q/D95Q}$-*rpoN* SI controller showed partial nitrogenase activity at ~50% of the WT strain in nitrogen free conditions (*Figure 3.6*). This activity supports previous findings and the results of the P*nifH* reporter assay that the GAF domain of NifA plays a role in regulating the activity of the transcription factor in response to cellular nitrogen conditions[86,130]. Nevertheless, the nitrogenase activity of the *nifA*$_{L94Q/D95Q}$-*rpoN* SI controller

demonstrates robust control of nitrogen fixation in an associative diazotroph in response to a biologically relevant trans-kingdom signal.

NifA is a σ^{54}-dependent enhancer binding protein, which in conjunction with σ^{54} RpoN, directs the binding of the polymerase to specific promoters and mediates the melting of the DNA duplex[84,257]. The NifA protein contains a highly conserved central AAA+ domain which uses the energy derived from ATP hydrolysis to facilitate DNA strand unwinding and σ^{54} complex formation and is essential for initiation of transcription by NifA[83,258]. The C-terminal domain of NifA also contains a highly conserved HTH domain which binds the upstream activator sequence which is conserved across diazotroph *nif* genes[259]. It is the N-terminal GAF domain which is involved in regulation of NifA protein activity in response to environmental factors[82,130]. In symbiotic systems, oxygen status is the dominant regulator of NifA activity, whereas nitrogen status is the major factor in free-living diazotrophs[45].

In addition to transcriptional regulation of *nifA*, which is abolished in our rhizopine controller system, post-transcriptional regulation of NifA has been documented. However, this primarily occurs in non-symbiotic strains which also encode the NifL protein[86,149,150]. For example, in the free living diazotroph *Klebsiella pneumoniae* NifL inhibits NifA activity in the presence of oxygen through binding of an FAD cofactor, or if cellular levels of fixed nitrogen exceed a threshold through binding of the NifA GAF domain[148-150]. The expression of *nifL* itself is regulated by NtrC is response to low nitrogen conditions, allowing integration of multiple regulatory signals at the transcriptional and protein level[151,212]. In *A. vinelandii,* the NifA GAF domain binds 2-oxo-gluterate as a measure of cellular nitrogen conditions, thereby preventing GAF domain binding by NifL[148,149,153,214]. 2-oxo-gluterate binding abolishes oxygen repression of nitrogen fixation which enables *A. vinelandii* to perform fixation under aerobic conditions, alongside a suite of nitrogenase oxygen protection mechanisms[44,260]. Whilst in *R.*

capsulatus the NifA protein belongs to a class of oxygen sensitive proteins which are independent of NifL[139]. In the free living diazotroph *A. brasilense* the PII protein encoded by *glnB* is required for the regulation of fixation in response to the nitrogen status through post-translational modification of NifA[209]. The homologue of NifL is not present in rhizobia species, therefore the role of the NifA GAF domain remains to be elucidated[46].

Studies into the partial deletion of the GAF domain in *S. meliloti* abolished NifA function, possibly due to protein instability or misfolding[82]. *A. caulinodans* likely employs regulatory systems for NifA which combines both symbiotic and free living control mechanisms found in other diazotrophs, as it is able to fix under both conditions. The inactivation of *A. caulinodans* NifA in free living N_2 fixation under high nitrogen conditions suggests negative regulation by currently unidentified protein[125,130,261]. This regulatory mechanism may be through a protein which is functionally homologous to NifL, or through regulation mediated directly by PII proteins in response to fixed nitrogen as seen in *A. brasilense*. Deletion of the NifA GAF domain in *A. caulinodans* abolishes its activation of *nif* genes[45], whilst the mutation of two residues as demonstrated here is enough to partially restore activity under high nitrogen conditions[99]. Though further regulation may have been imparted directly on the nitrogenase complex under these conditions preventing a full rate of nitrogen fixation. Further work is required to elucidate the mechanism the GAF domain plays in regulating NifA activity under nitrogen fixation conditions in *A. caulinodans*.

Despite developing a NifA controller which is partially insensitive to nitrogen regulation, no ammonia secretion into the growth media was observed using the indophenol method in WT or engineered *A. caulinodans* strains induced with SI under microaerobic conditions[262]. Lack of ammonia in the assay culture suggests that despite removing hierarchical regulation of the *nifA* transcription factor, fixed nitrogen was still assimilated by the cell[23,129]. Despite demonstrating control of free living fixation in our engineered *A. caulinodans* strains,

development of nitrogen secretion under fixation conditions is also required to make them a viable agricultural inoculant.

Control of nitrogen fixation and assimilation in response to the nitrogen status is incredibly important in free living diazotrophs as they are not benefiting from a plant host's symbiotic environment and supply of carbon and therefore must carefully balance the energetic demands of fixation with the cell's nitrogen requirements. *A. caulinodans* possesses two PII homologues, *glnB* and *glnK*, which may regulate different cellular targets[125,135]. Bacterial nitrogen assimilation occurs through the synthesis of glutamate from ammonium and 2-oxoglutarate, which is either catalysed by the enzyme glutamate dehydrogenase or the combined activities of glutamine synthetase (GS) and glutamate synthase (GOGAT). The GS enzyme, GlnA, is activated by deadenylation of each of its subunits by the adenylyl transferase GlnE which itself is activated via dephosphorylation by uridylylated PII proteins. PII proteins are required for NifA activity under nitrogen limitation in some bacteria, such as *A. brasilense,* though this is not the case in *A. caulinodans,* whereby GlnB and Glnk control the activity of NtrB and NtrC which regulates the transcription of *nifA* under free living conditions. However, *A. caulinodans* PII proteins may additionally inhibit NifA activity under high nitrogen conditions, as the characterization of ammonium-tolerant $NifA_{L94Q/D95Q}$ mutant indicates that the N-terminal domain of NifA was involved in post-translational regulation[46,129].

It has previously been reported that insertional inactivation of both *glnB* and *glnK* in *A. caulinodans* led to high levels of GS inactivity due to the mutant's inability to deadenylylate GS[129]. Deletion of *glnB* and *glnK* also prevented feedback inhibition of nitrogenase activity by ammonia and resulted in ammonium secretion under free living nitrogen fixation conditions[129]. In an attempt to replicate this phenotype we attempted to produce a *glnB glnK* double mutant via markerless deletion of *glnB* and replacement of *glnK* with a spectinomycin cassette (Spec) using the pK19mobSacB suicide plasmid[254]. Single gene knockout of *ΔglnB* or *ΔglnK::Spec*

could be generated by selecting for double crossover deletion or replacement[126]. However, despite successive attempts, we were unable to select for a subsequent double mutant on media supplemented with glutamine as a nitrogen source which suggests one or both proteins are required for growth, and that a double mutant resulted in a lethal phenotype. The role of PII proteins in bacterial growth is supported by attempts to construct in *glnK* mutant in *A. vinelandii* resulting in a lethal phenotype, and previously reported double *glnB glnK* mutants in other bacteria being extremely growth impaired[126,263]. Single gene mutants of either *glnB* or *glnK* did not result in ammonia secretion, suggesting both PII homologues are required for GS inactivation[126]. The double *ΔglnB ΔglnK::Spec* phenotype likely resulted in insufficient GS activity preventing cellular growth and possible disruption to other essential cellular functions, thus requiring an alternative strategy to induce ammonium secretion. Instead, control of ammonium secretion was developed by Dr T Haskett by controlling expression of a unidirectional adenylyl transferase by P*nifH* in an *A. caulinodans ΔglnE* strain resulting in strong GS inactivation and abolishment of feedback inhibition of nitrogenase[126]. Coupled with induction of the $nifA_{L94Q/D95Q}$-*rpoN* controller in the same strain, this linked inducible nitrogen fixation with shutdown of GS for ammonia secretion under control of SI induction in microaerobic conditions.

Furthermore, an *in situ* assay for measurement of plant-associative nitrogen fixation was developed by Dr T Haskett[249]. This assay allowed measurement of nitrogenase activity of SI controller strains developed in this work on the roots of *RhiP* barley. Using *A. caulinodans* carrying the P*mocB::*$nifA_{L94Q/D95Q}$-*rpoN* (pOPS1476) we were able to demonstrate 15% and 5% effective nitrogenase activity on T1 and T2 *RhiP* barley respectively, relative to WT associative fixation at 3% atmospheric oxygen. The difference in activity suggests control of nitrogenase induction by the host plant is dependent on the rhizopine concentration excreted from the roots, however subsequent flow cytometry of GFP fluorescence in bacterial root-

associated populations found only 3.6% of cells from T2 *RhiP* plants and 4.96% of cells from T1 plants were induced by the plant derived SI signal[201]. Furthermore, only 3% of bacterial colonies recovered from T2 *RhiP* roots retained the capacity to respond to SI when re-cultured in media, and the SI controller strain was only 50% as effective at colonising the root surface and endosphere relative to the WT[248]. Together, these results demonstrate our engineered SI controller strains were able to colonise the roots of cereals and fix nitrogen in response to rhizopine supplied by the plant host, however, genetic silencing of controllers and reduced colonisation ability may have reduced the overall effective nitrogenase activity in the engineered system versus WT *A. caulinodans*. Further optimisation of bacterial rhizopine signalling circuits and secretion of rhizopine by the plant host will be required to improve the control and efficiency of associative nitrogen fixation in this system. Greater induction of recipient engineered strains is required alongside a reduced fitness cost of rhizopine sensing in order for N_2 fixing strains to be used in real world applications.

Chapter 4

Control of Symbiotic Nitrogen Fixation in *Sinorhizobium meliloti*

4.1 Overview

The results of Chapter 3 demonstrate that control of nitrogen fixation in a plant host dependent manner is possible, however the efficiency of the engineered associative system was greatly decreased compared to WT *A. caulinodans* fixation. Despite the demonstration of binary control of nitrogen fixation *in vitro*, the same *A. caulinodans* P*mocB::nifA*$_{L94Q/D95Q}$ strains only showed partially effective nitrogen fixation on SI producing barley lines[201]. The decrease in nitrogenase activity was potentially due to genetic silencing of rhizopine circuitry, as recovered root associated bacteria from T2 *RhiP* barley only showed 3% of re-cultured cells were able to respond to rhizopine[248]. Moreover, *A. caulinodans* carrying the SI NifA controller were mildly defective for root-colonisation compared to WT *A. caulinodans*. Subsequent flow cytometry analysis of induction of root associated SI NifA controller strains on *RhiP* barley showed that only 5% of the bacterial population were induced by plant rhizopine secretion[201]. Together, this likely led to the decreased nitrogen fixation seen in the engineered system.

Building on this demonstration of rhizopine control of nitrogen fixation will likely require further optimisation of the rhizopine biosensor to achieve a greater level of SI perception within a bacterial population and the engineering of a more efficient synthetic symbiosis to deliver stable nitrogen fixation for agricultural use. This symbiosis will likely take the form of engineered barley nodules, where a protected microaerobic environment colonised by an engineered rhizobial strain could be coupled with rhizopine signalling circuitry to deliver fine-tuned control of bacterial fixation and more efficient nutrient exchange with the host.

The rhizobium-legume symbioses which forms the model of symbiotic nitrogen fixation is a relatively recent evolutionary adaptation, which has co-opted mechanisms from the much more ancient endosymbiotic symbiosis formed by arbuscular mycorrhizal (AM)

fungi[30,33,223]. Because of the close evolutionary distances, it has been found that rhizobial Nod factors which are formed from lipochitooligosaccharides (LCOs) are structurally similar to the Myc-factor signals used in AM symbiosis[218]. Both LCO signals bind Lysin-motif receptors and active a common SYM pathway leading to bacterial partner recognition and organogenesis of root nodules[34,50], for example in *M. truncatula* three genes, *DMI1*, *DMI2*, and *DMI3*, which transduce the Nod-factor signal from rhizobia are required in both nodulation and AM symbiosis[264]. The SYM pathway is conserved throughout cereal species, and therefore provides an opportunity to engineer Nod factor perception and activation of the SYM pathway for recognition of nitrogen fixing bacteria into cereals. The engineering of nodulation into cereals presents a monumental challenge and the degree of bacterial colonization which will result is unknown.

Typically, legume nodulation proceeds after recognition through root hair infection thread formation initiating nodule primordia formation through cell division within the root inner cortex, endodermis, and pericycle layers[53,61]. However, even rudimentary nodulation, which can form through less stringent root hair independent infection mechanisms such as crack entry, are still used by 25% of all the legume genera and is even seen with endophytic *Bradyrhizobium* ORS278 in *Oryza sp*[9,265]. Root hair independent methods of rhizobial entry still result in delivery of fixed nitrogen to the host and may be a more realistic target of engineering fixation in non-legumes.

Plant hormones, such as cytokinin and auxin regulate several developmental processes in plants. Cytokinin signalling is likely to act in concert with auxin to initiate nodulation, with research showing that cytokinin receptor *CRE1* in *M. truncatula* is a prime target of the SYM pathway and leads to induction of symbiosis-specific transcription factors such as *Nodule Inception* (*NIN*) which are required for nodule organogenesis[26,80,177]. It is likely that cytokinin

induces local auxin biosynthesis and changes in polar auxin transport to induce nodule development in a similar manner to the progression of lateral root development[59].

The high degree of overlap in nodule and lateral root development can also be leveraged for engineering efforts to transfer nodule organogenesis to cereal species[218]. Characterised components from cytokinin induction of root organogenesis could be employed instead of engineering the entire legume nodulation developmental program. For example, treatment of *Medicago* or cereal roots by phytohormones can trigger cortical cell division leading to 'nodule like structures' (NLS) even in the absence of bacteria (*Figure 4.1*)[185]. If NLS could be induced on intended cereal hosts and coupled with rhizopine biosynthesis, then a system for engineering control of a closer and more efficient symbiotic system could be developed. Our work aimed to show this was a viable strategy to improve upon the control of associative symbiosis discussed in Chapter 3. By using *RhiP M. truncatula* and SI signalling circuitry in the symbiotic partner strain *S. meliloti* to demonstrate improved induction and control of fixation by rhizopine in a nodule environment.

Figure 4.1 Image of *H. vulgare* nodule-like structures after treatment with 0.5mg L^{-1} of 2,4-dichlorophenoxyacetate. Image taken by Dr Timothy Haskett.

Another key aspect of engineering biological nitrogen fixation for agriculture will be to overcome the mechanisms of negative regulation on the genes and proteins required for nitrogenase gene induction and activity. Fixing atmospheric nitrogen by a molybdenum dependent nitrogenase requires expression of up to 16 *nif* genes in *K. oxytoca* and *A. vinelandii,* as well as several additional accessory genes for its assembly and function[266]. *Nif* gene expression, coupled with oxygen protection mechanisms, and electron transport to the nitrogenase complex can result in up to 42 mol of ATP per mol of N_2 fixed[155,200,206]. Diazotrophs have therefore evolved stringent regulation of nitrogen fixation to conserve energy, primarily in response to cellular O_2 and NH_3, as seen in with the negative regulation of WT *A. caulinodans nifA* in Chapter 3.

Recent advances in synthetic biology allows for the domestication and transfer of *nif* gene clusters on broad-host range plasmids between rhizobacteria[267,268]. Transfer of engineered gene clusters may help to overcome native regulatory factors as they may be absent in the recipient bacterium. Compatibility of *nif* clusters was first demonstrated through the transfer of the *K. oxytoca* M5a1 *nif* cluster to *E. coli*, conferring the ability to fix nitrogen[202]. Research in the transfer of *nif* clusters has demonstrated that the effectiveness of gene expression and nitrogenase activity in the recipient strain is strongly influenced by the genotype and therefore the relatedness of the donor versus the recipient. A minimal *nif* gene clusters was transferred from *Paenibacillus polymyxa* WLY78 to *E. coli* resulting in 10% activity of that observed in WT *Paenibacillus*[269]. Nitrogenase activity was further improved by inclusion of electron transport genes *pfoAB-fldA* and nitrogenase Fe-S cluster assembly genes *nifSU*, which were lacking in the *E. coli* host, resulting in 50% activity[203]. Furthermore, the transcription of *nif* genes in *Paenibacillus* was inhibited by a high concentration of ammonia and by external oxygen, whereas placing transcription of the *nif* genes under the native *Paenibacillus* σ^{70} dependent promoter led to constitutive expression in *E. coli*. These results indicate that the

negative regulation of *nif* gene transcription and activity was absent in the heterologous host[203,206].

Further evidence of transfer of *nif* clusters leading to circumvention of native ammonia repression of nitrogen fixation comes from the transfer of *nif* clusters between *Pseudomonas* species. The nitrogen fixation island from *P. stutzeri* A1501 was transferred to *P. protogens* Pf-5 via a recombinant cosmid resulting in nitrogen insensitive constitutive nitrogenase activity and ammonium release by the engineered strain[207]. Furthermore, this phenotype was also observed in *Pseudomonas putida*, *Pseudomonas veronii* and *Pseudomonas taetrolens* transformed with the recombinant *nif* cosmid; but not in *Pseudomonas balearica* and *Pseudomonas stutzeri* due to compatible ammonia regulation in these strains[207]. Transfer between these strains demonstrates that the adaptability of nitrogenase expression and activity is dependent on the host genome. The role of the host genome is also seen in the transfer of *nif* clusters between more distantly related bacteria, where the transfer of recombinant *nif* genes between alphaproteobacteria and gammaproteobacteria resulted in no activity, potentially due to transcriptional incompatibility[99]. Therefore, despite the advances in synthetic biology allowing the transfer of *nif* clusters between strains, the genomic background and the relatedness of the recipient are key factors in determining the success of producing effective nitrogen fixing strains.

Refactoring *nif* gene clusters by modular combinatorial design provides a further strategy to overcome native regulation of gene clusters, even when not all the regulatory elements present are known. Refactoring a gene cluster involves placing multi-gene pathways from the host under the control of synthetic genetic sensors and circuits. These genetic parts enable the *nif* cluster to be controlled in an inducible manner and eliminates the influence of native cellular or environmental stimuli which can repress gene activity. All noncoding DNA and regulatory genes are removed, and the codons of essential genes are changed to be as

divergent as possible whilst still encoding the correct gene product, thus removing unknown internal regulation such as operators, promoters, mRNA secondary structure, pause sites, methylation sites, and codon regulation. The recoded genes are organized into synthetic operons whose transcription are controlled by characterised synthetic parts: promoters, ribosome binding sites, and terminators, for optimal gene expression and to maintain the required protein stoichiometry (*Figure 4.2*)[204,205,266,267].

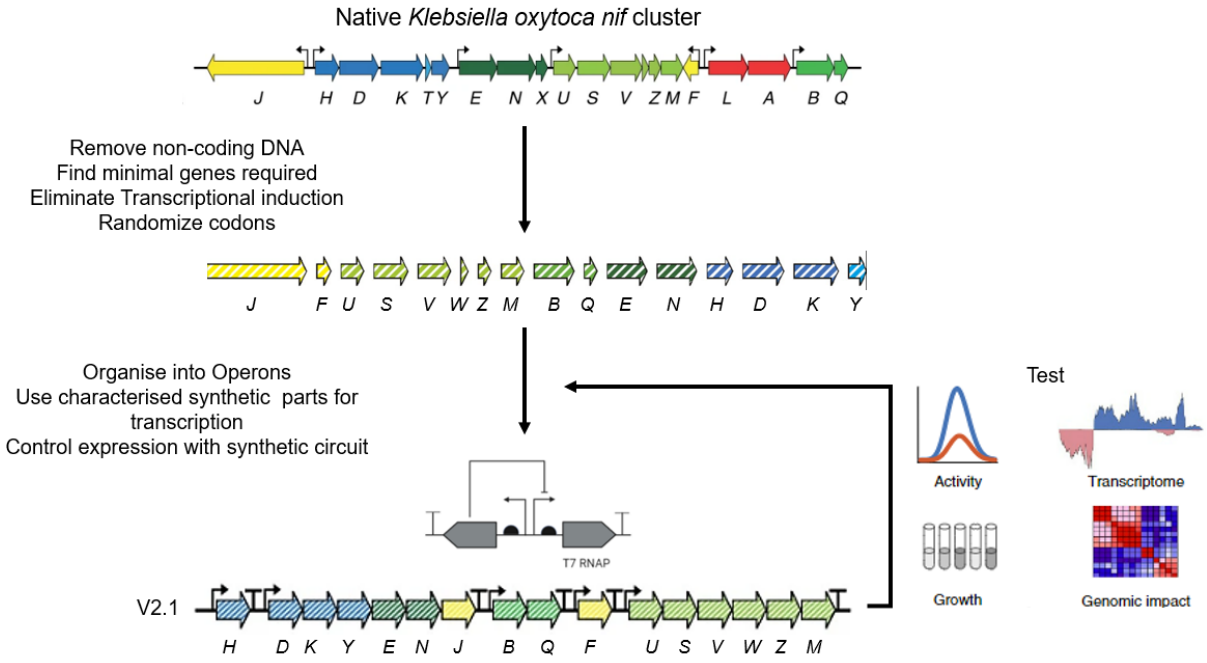


Figure 4.2 Diagram of the design of the refactored *nif* cluster from *K. oxytoca.* The native cluster is shown (top), the genes are coloured by function: blue (nitrogenase), green (cofactor biosynthesis, shading corresponds to operons), yellow (e^- transport), and grey (unknown). Native regulation of genes is removed and replaced by synthetic parts, genes are organised into operons and their activity tested by induction from a synthetic controller (shown in grey) before iterative design cycles informed by testing. Figure adapted from *Temme et al*, 2012[204].

This approach was applied to the *nif* gene cluster from *K. oxytoca* which is encoded by 20 genes organised intro 6 operons on 23.5Kb of DNA[202]. Like most diazotrophs *K. oxytoca*

fixes nitrogen by a molybdenum-dependent nitrogenase, which is composed of two proteins, MoFe protein and Fe protein. The MoFe protein component is a heterotetramer encoded by *nifD* and *nifK* that contains two metalloclusters: FeMo-co, a Molybdenum-Iron-homocitrate cluster which serves as the active site of substrate binding and reduction, and the P-cluster, an iron-sulfur cluster which shuttles electrons to FeMo-co. The Fe protein, encoded by *nifH*, is a homodimer which contains a 4Fe-4S cluster which donates electrons to the MoFe protein. The assembly pathway for nitrogenase is complex and requires several genes for metal cluster biosynthesis, in addition to other gene products necessary to produce a fully functional enzyme. Therefore, alongside the structural genes, *nifY, E, N, U, S, V, W, Z M, B, Q* are required for the synthesis and processing of the FeMo-co metallocluster, and the nitrogenase structural subunits. A further two genes *nifJ* and *nifF* code for specific components of the electron transfer pathway from pyruvate to the Fe-protein subunit of nitrogenase[43]. Genes not included in the refactored cluster include the negative regulator *nifX,* and *nifT* whose gene product has no observed effect on nitrogen fixation[270,271]. The native cluster further encodes genes for the NifA-NifL regulatory system which represses *nif* gene expression under conditions of nitrogen excess and therefore impede activity of the refactored cluster[46]. The remaining 16 *nif* genes were organised into synthetic operons and placed under control of a T7 RNA polymerase specific promoter[204]. Use of a controller encoding T7 RNA polymerase to drive *nif* cluster gene expression provides tight regulation and a large dynamic range and is transcriptionally orthogonal from the host[272].

The initial refactoring of the *K. oxytoca nif* gene cluster, when transferred into *E. coli,* resulted in 10% nitrogenase activity compared to WT *K. oxytoca* activity[204]. Further combinatorial assembly was used to test operon variants and the resulting activity used to inform iterative designs to produce a variant *nif* cluster v2.1 which produced 57% nitrogenase activity relative to the WT strain[205]. However, transfer of the v2.1 cluster alongside a T7 RNA

polymerase controller into the cereal endophytes *P. protogens* Pf-5 and *Rhizobium sp* IRBG74 did not lead to nitrogen fixation. The inactivity of the cluster appeared to be due to less active promotors and terminators in the rhizobial hosts resulting in reduced expression and different translation rates[99]. Based on this, a second refactored *K. oxytoca* cluster v3.2 was made with stronger promotors and terminators and preserved host operon structure for better expression ratio of gene products. The optimised v3.2 cluster resulted in reduced activity of nitrogenase in *E. coli* but v3.2 showed nitrogenase activity in both *P. protogens* Pf-5 and *Rhizobium sp* IRBG74[99].

We can capitalise on this development by testing these refactored *nif* clusters in a host rhizobium to first demonstrate free living fixation and potentially ammonium secretion, followed by developing the strain for *in planta* delivery of nitrogen fixation. Alongside the *P. protogens* Pf-5 and *Rhizobium sp* IRBG74 strains used by Min Ryu *et al,* we can also test these clusters in model symbiotic strains like *S. meliloti* to see if refactoring genes can lead to ammonia insensitive controllable nitrogen fixation in a symbiotic strain. We can first demonstrate control of fixation using characterised chemically inducible sensors to drive T7 RNAP expression, before developing a controller based on a more biologically relevant signal such as the rhizopine trans-kingdom signal engineered into *RhiP* barley.

4.2 Use of Refactored *nif* Clusters in the Symbiotic Rhizobium *S. meliloti*

4.2.1 Testing Repressor Promotor Pairs in *S. meliloti*

As previously shown, plasmid controllers provide a simple tuneable input to regulate the expression of gene clusters. By testing these sensors in a rhizobial strain we can develop a system to control expression of *nif* genes from engineered gene clusters in response to environmental signals. The symbiotic strain *S. meliloti* was used to test the plasmid sensors, before developing an optimised controller to test the refactored *K. oxytoca* nitrogen fixation

cluster v2.1, which conferred nitrogen fixation to *E. coli*, and v3.1, which conferred nitrogen fixation to IRBG74 (both supplied by Min Ryu[99]). Five inducible promotors fused to *gfp* were tested for tight regulation and good dynamic range in *S. meliloti* strain CL150. The sensors were controllable by natural signals such as flavonoids, as well as synthetic signals typically used in molecular biology such as IPTG. All biosensors were carried on low copy number RK2 plasmids and conjugated into *S. meliloti* by tri-parental conjugation with *E. coli* carrying helper plasmid pRK2013. *S. meliloti* sensor strains were grown for 20 hours in N rich minimal media (UMS supplemented with 20 mM succinate and 10 mM ammonium chloride) in triplicate with a range of inducer concentrations before endpoint measurements of fluorescence were taken.

The first biosensor tested was the naringenin inducible GFP reporter plasmid pOPS1536 in CL150 strain OPS2632, which encodes the *S. meliloti* 1021 P*nodA* promotor on a low-copy number plasmid. In the native system, the *nodA* promotor is typically regulated by LysM-family of NodD proteins which activates transcription of nod genes, required for LCO biosynthesis and symbiosis, in the presence of flavonoids. The P*nodA* sensor showed high sensitivity to naringenin with induction as low as 10 nM and had a large dynamic range of $5x10^4$ over the inducer concentrations tested, however, it had the highest basal level of induction of the biosensors tested (*Figure 4.3*). Another rhizobial specific promotor tested was P*tau*, also from *S. meliloti*, in plasmid pLMB509 which was previously constructed in this lab[219]. The pLMB509 plasmid was conjugated in CL150 to produce strain OPS2631. The P*tau* promotor is from an ATP binding cassette (ABC) transporter that is positively regulated by TauR in conjunction with the amino acid derivative taurine[219]. The taurine inducible biosensor only showed 10-fold induction over the baseline and required 20 mM inducer to achieve this, it also had a very low hill slope coefficient of 0.45 demonstrating loose regulation by taurine (*Figure 4.3*).

A third natural product biosensor tested was P*phlA* in plasmid pOPS1537, which was conjugated into CL150 (OPS2630). The P*phlA* promotor is from *Pseudomonas fluorescence* which produces the secondary metabolite antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) during colonisation of the rhizosphere. The promoter controls expression of the 2,4-DAPG biosynthesis operon *phlACBD* and is regulated by the PhlF tetR-like repressor which binds to the *phlO* operator site. To ensure tight inhibition in the absence of 2,4-DAPG by PhlF, which is divergently encoded on the same plasmid, the *phlF* gene is constitutively expressed from the P*lacIQ* promoter. The pOPS1537 plasmid produced a large dynamic range of *gfp* expression in *S. meliloti* with induction at 10 μM producing a 10^4-fold induction over the baseline and with tight regulation by PhlF giving a hill slope of 3 (*Figure 4.3*). A similar result was seen with plasmid pOPS1577 in CL150 (OPS2627), which constitutively encoded the *tetR* repressor under P*lacIQ* for transcriptional activation of expression of *gfp* from P*tet* in response to the anhydrous tetracycline (aTc) inducer[273] (*Figure 4.3*). The pOPS1577 plasmid showed a similar dynamic range but greater sensitivity that pOPS1537, with *gfp* induction seen at 50 nM aTc. Finally, an IPTG inducible plasmid pOPS1631 was tested in CL150 (OPS2629). The plasmid pOPS1631 encodes the tightly regulated P*lac* derivative promoter P*A1lacO1* controlled by the divergent constitutive *lacI* repressor[274]. Although this plasmid showed the lowest maximum induction, it displayed tight regulation of *gfp* expression with low basal expression and 10-fold induction seen with 1mM IPTG (*Figure* 4.3).

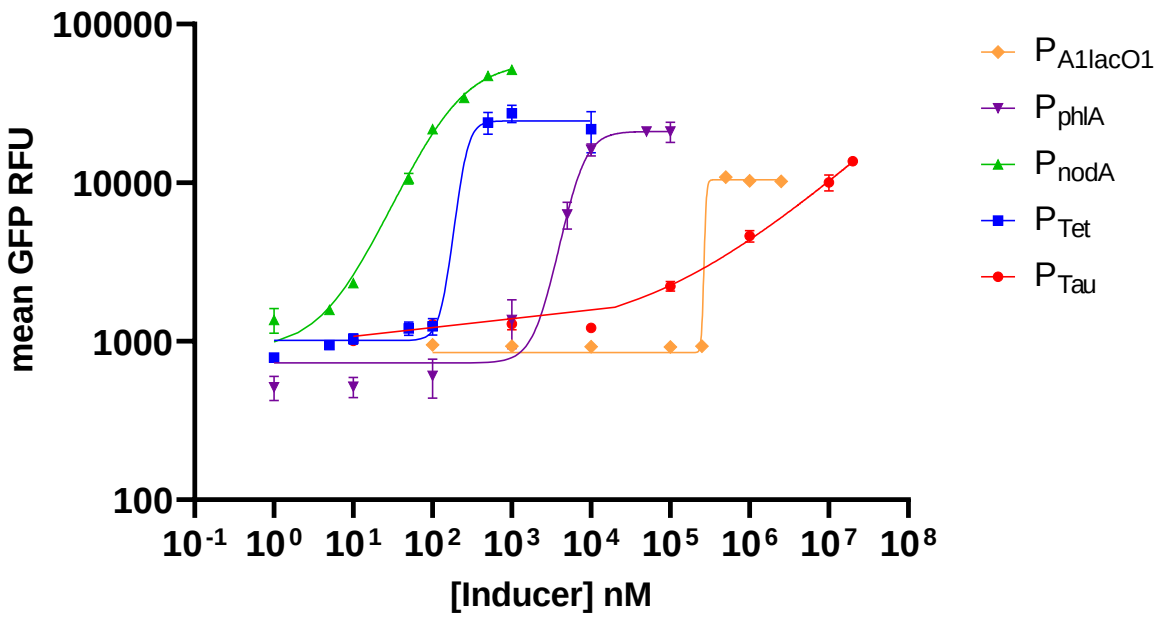


Figure 4.3 Mean activity of repressor-promoter pairs in *S. meliloti* CL150 (± SEM, n = 3). Expression of *gfp* from *lacI*-P*A1lacO1* is shown in orange, *phlF-PphlA* in purple, *nodD-PnodA* in green, *tetR-Ptet* in blue, and *tauR-Ptau* in red. Cultures were grown in minimal media supplemented with 10 mM NH_4 and 20 mM succinate and induced for 18 hours at 28°C before end-point measurements of *gfp* induction and OD_{600} were measured via plate-reader.

4.2.2 Construction of T7 RNA Polymerase Controller and Reporter

To drive expression of the refactored *Klebsiella nif* clusters, an IPTG inducible T7 RNAP controller pZH19 was obtained from the Voigt lab[99]. The plasmid controller contained the repressor *lacI* driven by the strong constitutive promoter P*lacIq,* as well as T7 RNAP expressed from P*A1lacO1*, a strongly repressed yet highly inducible promoter which is orthogonal in *S. meliloti*[274]. The P*lacIq* is ideal for induction of refactored clusters as it was previously shown that increasing the inducer concentration above a maximum led to a decline in cluster activity. The controller was assembled into a pRK415 backbone which is a medium

copy vector which lacks *PAR* genes, which would be incompatible with the P*MQ131PAR* vector used to construct the refactored *K. oxytoca* clusters.

Alongside construction of a T7 controller, A reporter plasmid was constructed using golden gate cloning using previously cloned genetic parts[247]. The reporter was constructed using pBBR1 vector backbone pOGG024 and combined P*T7* from pOGG152, RBS_{std} from pOGG143, *sfGFP* from pOGG037, and T7 terminator from pOGG039 using golden gate cloning[275,276]. The constructed reporter plasmid (pOPS1716) was sequenced using primers oxp0284/0283.

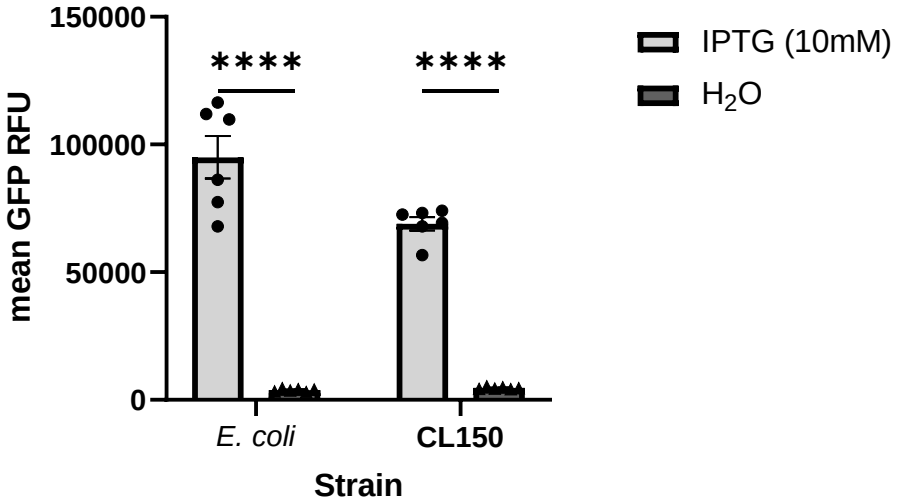


Figure 4.4 Induction of T7 RNAP controller in *E. coli* and CL150 strains carrying pZH19 and pOPS1716 P*T7::gfp* reporter. Strains were grown on M9 minimal media and induced with 10 mM IPTG for 20 hours. (± SEM, n = 6, independent two-tailed Student's T-test, **** $p \leq 0.0001$).

Both the T7 controller (pZH19) and reporter (p1716) were conjugated into *E. coli* DH5α and *S. meliloti* CL150 (OPS2634) to test gene expression by T7 RNAP. Both strains were grown in minimal media (M9 supplemented with 20 mM succinate and 10 mM NH_4Cl) for 20 hours with expression induced by 100 μM IPTG. The IPTG inducible T7 controller pZH19

produced strong induction of the P*T7* in both strains, with induction in *E. coli* 25% greater than in CL150, with over 10^4-fold induction over the uninduced state in both strains, demonstrating tight regulation of P*A1lacOI* expression by LacI (*Figure 4.4*).

4.2.3 Testing Induction of Refactored Clusters in Rhizobium

After confirming expression of T7 RNAP by the controller plasmid pZH19 in both *E. coli* and CL150, the controller was conjugated into bacteria alongside the refactored clusters v2.1 and v3.2. The bacterial strains used for expression of the refactored *nif* clusters were *S. meliloti* CL150 Δ*nifA* OPS1507 (producing OPS3106 and OPS3107), *E. coli,* the cereal epiphyte *P. protegens* Pf-5 (producing OPS3537 and OPS3538), which does not contain *nif* genes but has previously demonstrated successful transfer of *nif* genes[96,202,269,277], and the cereal endophyte *Rhizobium* sp. IRBG74 Δ*nif*Δ*hsdR* (producing OPS3507 and OPS3508) which contains a deletion of both *nif* clusters and therefore does not fix nitrogen under free living conditions[99]. Both *P. protegens* Pf-5 and *Rhizobium* sp. IRBG74 were shown to fix nitrogen upon induction of *nif* genes from v3.2 cluster using a T7 controller, but not with v2.1[99]. The strains containing transferred *nif* clusters and pZH19 controller were cultured and evaluated for nitrogenase activity using an acetylene reduction assay. *E. coli* strains were cultured in minimal M9 media containing nitrogen overnight before washing with PBS and resuspending the culture at OD_{600} 0.4 in M9 N-free media. CL150, Pf-5, and IRBG74 were similarly cultured overnight in UMS containing nitrogen before washing and resuspending at OD_{600} 0.4 in N-free media. Although the refactored clusters should not be subject to regulation by heterologous strains, the N-free media would prevent any unknown interactions which may repress nitrogenase in rhizobial strains, thereby given the maximal rate of fixation possible. All bacteria cultures were induced with 10 mM IPTG at 1% oxygen and incubated in sealed McCartney vials at 28°C with shaking, nitrogenase activity was assessed over 20 hours.

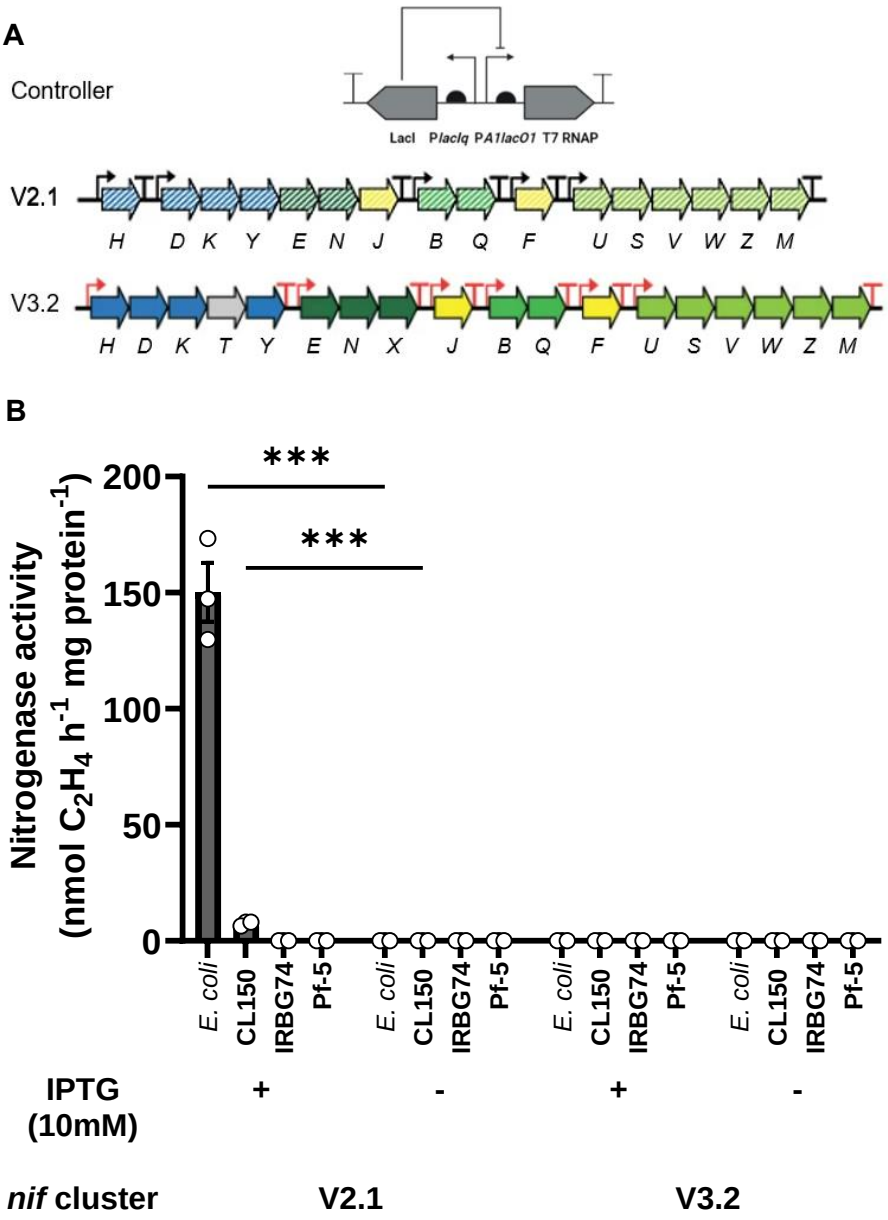


Figure 4.5 Assay for nitrogen fixation using refactored clusters **A.** Refactored *nif* clusters v2.1 and v3.2 with pZH19 T7RNAP controller used for induction of nitrogen fixation in bacterial strains. **B.** Nitrogenase assay of induced refactored *nif* cluster activity in four bacterial strains, *E. coli* DH5α, CL150 Δ*nifA, Rhizobium sp. IRBG74* Δ*nif*Δ*hsdR, Pseudomonas protegens Pf-5*. (±SEM, n = 3, multiple independent Student's T-tests, *** $p \leq 0.001$).

The refactored cluster v2.1 with the pZH19 T7 RNAP controller induced by addition of 10 mM IPTG showed nitrogenase activity in both *E. coli* and CL150 (OPS3106) (both p

≤0.001), though nitrogenase activity in *E. coli* was 20-fold greater (*Figure 4.5*). No fixation was seen with cluster v2.1 in *P. protegens* Pf-5 or *Rhizobium* sp. IRBG74 (OPS3507), as reported by Min Ryu et al, 2020. However, no activity was seen in any bacterial strain with refactored cluster v3.2, which was previously reported to function, albeit at low rates of fixation, in *P. protegens* Pf-5 and *R. sp* IRBG74[99]. As induction was seen with the same IPTG controller with cluster v2.1, this suggests an issue with the v3.2 plasmid.

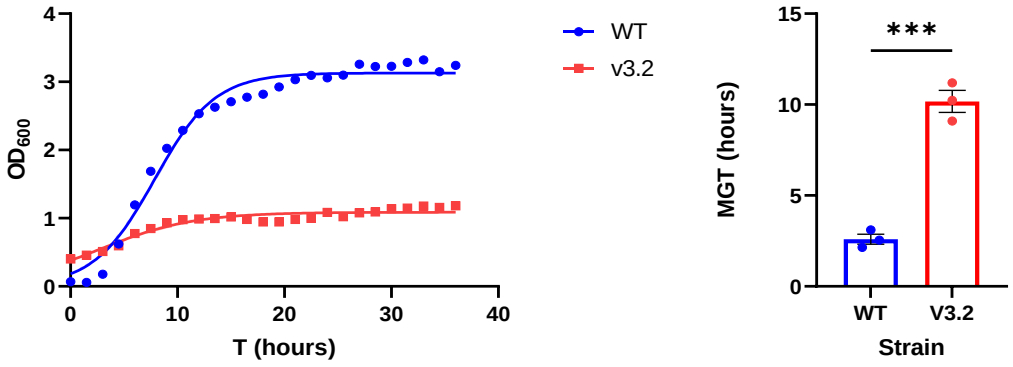


Figure 4.6 Left Representative growth curve of WT CL150 (blue) and CL150 Δ*nifA* v3.2 grown in TY media with shaking at 28°C. **Right** Mean generation time of WT CL150 and CL150 carrying the v3.2 cluster calculated from growth curves (± SEM, n = 3, independent two-tailed Student's T-test, *** p ≤ 0.001).

Growth of WT CL150 and CL150 carrying the v3.2 *nif* plasmid (OPS3107) was compared in TY media at 28°C. WT *S. meliloti* reached a maximal OD_{600} of 3 with a mean doubling time of 2.5 hours, whilst CL150 carrying v3.2, without induction, only reached an OD_{600} of 1 with a mean doubling time four-fold greater than the WT (10.17, p = 0.0003) (*Figure 4.6*). The increased doubling time suggests the v3.2 plasmid imposes a large metabolic burden on *S. meliloti* cells and impedes replication, even in nitrogen rich media and in the absence of IPTG inducer.

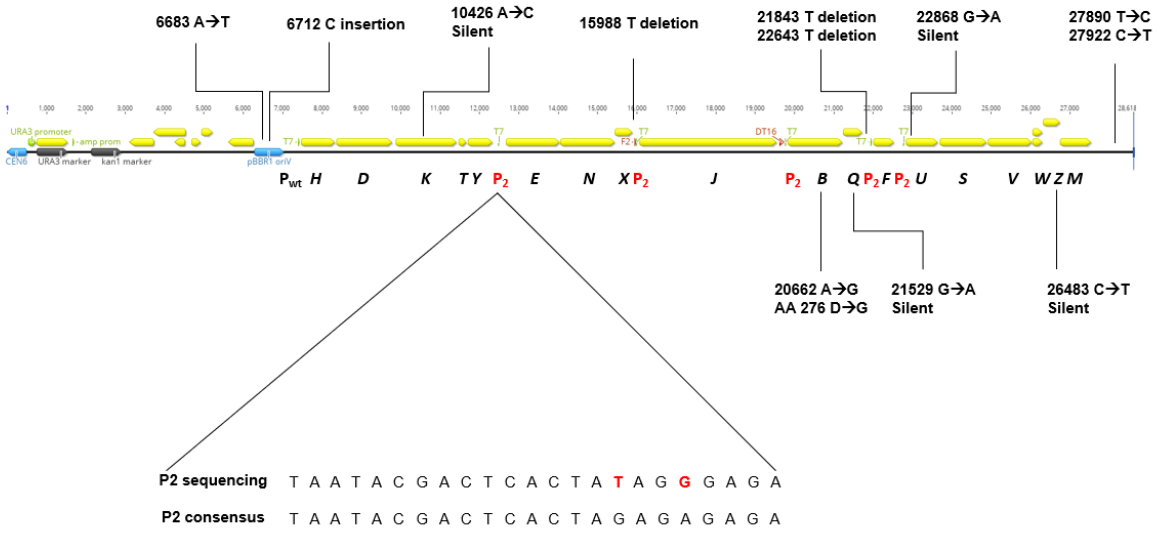


Figure 4.7 Representation of sequencing results of refactored cluster v3.2. Nucleotide changes and their base pair position are shown, with the effect of nucleotide substitutions in coding genes also described. The position of P2 promoters in the cluster are highlighted in red with the sequencing result compared to the consensus sequence showing two nucleotide changes expanded below.

To analyse the refactored clusters to see if any issues had arisen due to mutations, both v2.1 and v3.2 plasmids were sent for whole plasmid sequencing using Oxford Nanopore flowcell analysis. Sequencing results revealed several single-nucleotide polymorphisms (SNPs) in plasmid v3.2 which were not present in the functional v2.1 refactored cluster. Firstly, a transversion mutation at nucleotide 6683 and an insertion at nucleotide 6712 were identified which both may affect the pBBR1 oriV. In the plasmids the origin of vegetative replication (oriV) consists of direct repeats or iteron DNA sequences rich in A-T base pairs, this region is recognised by host Rep proteins to form the initial complex for initiation of plasmid replication[268,278,279]. SNPs in this region may affect the plasmid's ability to replicate which may cause some of the growth issues seen in CL150 strain OPS3107 carrying plasmid v3.2. Further SNPs were seen in *nif* genes *K, B, Q, U, and Z*, with most being silent mutations

with no change in the amino acid code (*Figure 4.7*). However, the transition mutation at nucleotide 20662 in *nifB* from A to G caused an amino acid substitution from aspartic acid to glycine (*Figure 4.7*). An amino acid substitution from an acidic R group containing residue to a neutral R group residue could affect the secondary and tertiary structure or the activity of the NifB protein. NifB protein is required for the biosynthesis of the iron-molybdenum cofactor (FeMo-co cluster) which serves as the active site of molybdenum nitrogenase found in *K. oxytoca*. Therefore, a mutation in this key enzyme catalysing an important step in nitrogenase formation may prevent formation of a functional nitrogenase complex, thereby abolishing nitrogen fixation. Lastly, the sequence result of the P2 promoters which are used to express five operons in v3.2 were found to contain two SNPs (*Figure 4.7*). These nucleotide substitutions, present in all the P2 promoters of sequenced v3.2, would likely alter the expression of these operons and therefore alter the ratio of *nif* gene expression which was rebalanced in v3.2 compared to v2.1. Further mutations were found outside of coding regions in the plasmid (*Figure* 4.7) indicating the plasmid was under larger evolutionary pressure due to the stress it imposed upon the cell leading to an accumulation of mutations which possibly disabled the *nif* activity of the plasmid.

4.3 Testing SI Biosensors in *S. meliloti*

4.3.1 Tuning SI Biosensors in *S. meliloti*

To assess if rhizopine biosensors were a viable option to control nitrogen fixation in the symbiotic *S. meliloti* strain CL150, the SI biosensors developed for induction in *A. caulinodans* were conjugated into CL150. As analysis of SI induction in *A. caulinodans* on roots of *RhiP* barley showed reduced plasmid stability and induction in a small subpopulation, alternative vectors were tested alongside CL150 carrying pBBR1 SI biosensor pOPS0889 (OPS2633). A medium copy number RK2-PAR vector, pOPS1052, was constructed in this lab using primers

oxp2750 and oxp2751 to amplify the rhizopine transport components (*intBC* and *mocB*) and perception loci (*mocR,* P*mocB::gfp*) from pOPS0889 for HiFi cloning into EcoR1 digested pOGG093, the resulting plasmid was conjugated into *S. meliloti* (OPS1726). A second R6K vector was also constructed for mini-Tn7 single-copy integration of the SI biosensor apparatus at an engineered *attB* site downstream of *glmS* in the CL150 chromosome (OPS1835)[248]. Rhizopine perception genes were amplified from pOPS0889 (oxp4954-53) and cloned into mini-Tn7 vector pOPS1744 digested with BamHI to give pOPS1740. The mini-Tn7 plasmid pOPS1740 was integrated into *S. meliloti* landing pad strain OPS1835 using tri-parental mating with helper plasmid pTNS3[280], produced *S. meliloti* strain OPS3099. Another strategy to reduce the toxicity of SI is to reduce the expression of *intBC* transporters, which accumulate SI in the cell, without reducing the perception genes as well. A series of previously characterised *Sinorhizobium* non-σ^{54} promoters were transcriptionally fused to *gfp* and screened for induction relative to the *J23115* Anderson promotor used in pOPS0889[89,248]. It was found that the promotor P*rpoD*, from the *Sinorhizobium* RpoD sigma factor, had four-fold lower expression than the synthetic P*J23115* previously used to express *mocBintBC*[248]. Therefore, a second pBBR1 plasmid was constructed with P*rpoD-intBC* amplified by oxp5876-77 and *mocRB*-P*mocB::gfp* amplified from pOPS0889 with oxp5878-79 and HiFi cloned into pMQ131-PAR digested with BamHI, producing plasmid pOPS1998. The resulting pBBR1 SI biosensor was conjugated into *S. meliloti* CL150 producing OPS3307 and the induction response to rhizopine compared to CL150 carrying pBBR1 plasmid pOPS0889.

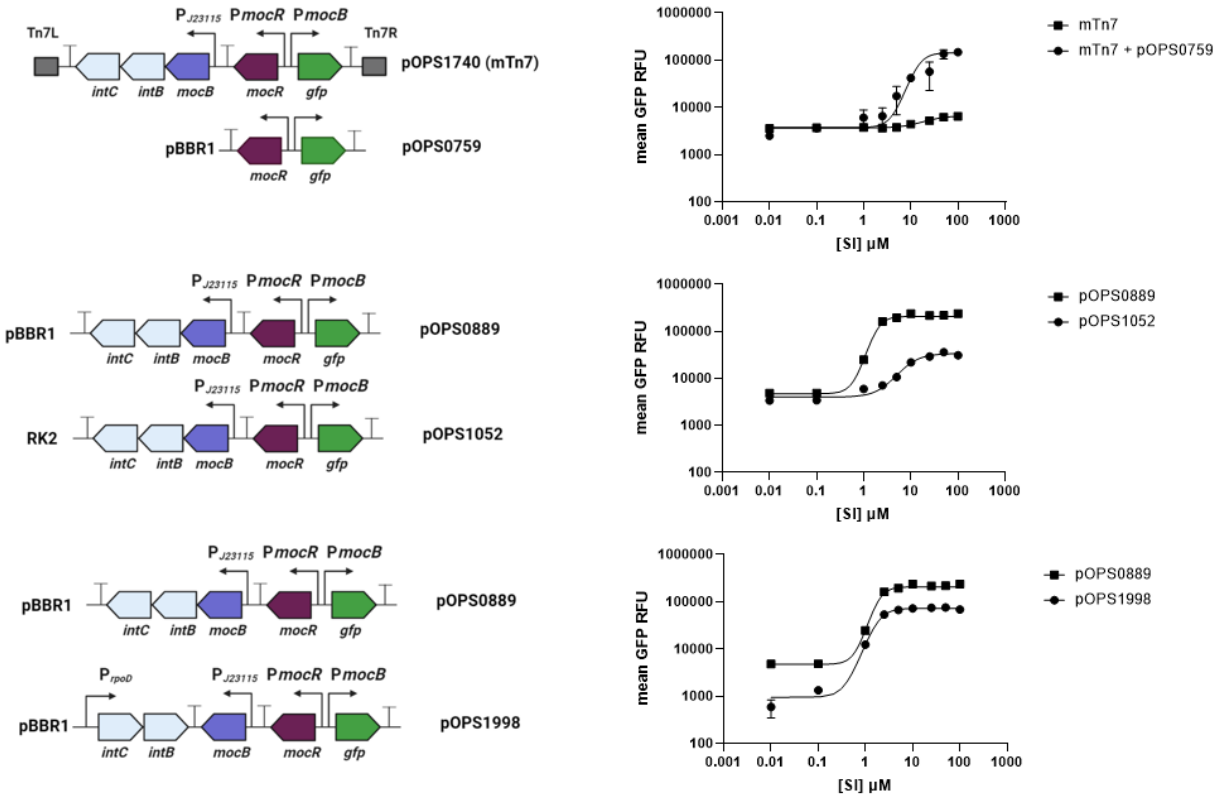


Figure 4.8 Induction curves of SI biosensors in *S. meliloti* CL150 grown in minimal medium and induced with SI for 18 hours expressed as mean GFP RFU ±SEM (n = 3). The SI biosensors and their replicons are shown on the left with their corresponding plasmid designation, pOPS1740 was integrated into the chromosome of CL150 at an engineered *attB* site. Plasmids pOPS1052 and pOPS1998 were compared to pBBR1 plasmid pOPS0889.

Firstly, the induction of pBBR1 SI biosensor pOPS0889 was tested in CL150. As seen in *A. caulinodans,* pOPS0889 produced a robust induction response to SI with 44-fold expression of *gfp* seen in CL150 at 10 µM SI (*Figure 4.8*). Strain CL150 with an integrated mini-Tn7 SI biosensor only produced a two-fold response over the range of SI concentrations tested. the lack of SI-inducible *gfp* expression may be due to insufficient levels of the MocR or MocB proteins, or potentially reducing the P*mocB::gfp* expression cassette to single copy. Therefore, the integrated SI biosensor was complemented with pOPS0759 carrying *mocR*-P*mocB::gfp* in strain OPS3101. Addition of the pOPS0759 plasmid resulted in 38.5-fold expression with maximal RFU seen at 100 µM SI which, although slightly reduced, was not

significantly different from induction with pOPS0889 (independent Student's T-test, p = 0.08) (*Figure 4.8*). SI perception was less sensitive at SI concentrations lower than 25μM relative to pOPS0889 ($p \leq 0.01$), likely due to the lower dosage of *intBC* transporters in the integrated strain. The RK2 biosensor pOPS1052 produced a 10.7-fold dynamic range across SI concentrations and produced a maximum RFU which was 6 fold lower than *gfp* expression seen with pOPS0889 ($p \leq 0.002$), reflecting the lower plasmid copy number of pOPS1052 (*Figure 4.8*). However, pOPS1052 still maintained sensitivity to SI with maximum induction seen at 10 μM . Lastly, the pBBR1 plasmid pOPS1998 with altered expression of transporters by P*rpoD*::*intBC* was still able to respond to SI, although perception was less sensitive at all SI concentrations than pOPS0889 ($p \leq 0.005$), presumably due to reduced transporter expression. However, pOPS1998 still maintained a larger dynamic range of *gfp* expression from P*mocB* which was induced 76.8-fold at 10 μM SI (*Figure 4.8*).

4.3.2 *S. meliloti* SI Biosensor Induction on *RhiP* Barley

To measure the stability of expression from these biosensors in CL150, the *in situ* SI dependent expression of these strains on *RhiP* barley was tested. Firstly, a CL150 strain expressing a constitutive fluorophore was constructed to allow detection of *S. meliloti* cells isolated from the roots of barley via flow-cytometry. The CL150 strain OPS1835 was used, as it encodes a chromosomal mini-Tn7 landing-pad, allowing integration of constitutive mCherry expressed from the strong constitutive Anderson promoter P*J23104* by conjugation with pOPS1531 and pTNS3 helper plasmid, producing CL150 constitutive mCherry strain OPS2879. CL150 OPS2879 was then conjugated with pBBR1 SI biosensor pOPS0889 (OPS3289), RK2 biosensor pOPS1052 (OPS3509), and P*rpoD* biosensor pOPS1998 (OPS3503). As strain OPS3101 already contained an integrated SI biosensor at the *attB* site and pBBR1 plasmid pOPS0759, it was labelled with constitutive mCherry by conjugation with RK2 plasmid P*J23104::mCherry* pOPS1879 to produce strain OPS3065.

T2 lines of SI producing barley were germinated and grown in 100 mL Schott bottles in sand according to the protocol developed by Haskett et al, 2021[249]. Barley seedlings were inoculated with CL150 strains to approximately $1x10^8$ cells and grown for 7 days post inoculation. The bacteria on the root surface were isolated by uprooting the plant, washing the roots in PBS, and grinding the roots using a Pestle and mortar. Flow cytometry was then used to assess single-cell P*mocB::gfp* induction in bacteria with mCherry expression greater than the 5000a.u threshold. Cells responding to SI were termed GFP+ and were measured relative to the 99th percentile of GFP fluorescence in cells on WT barley.

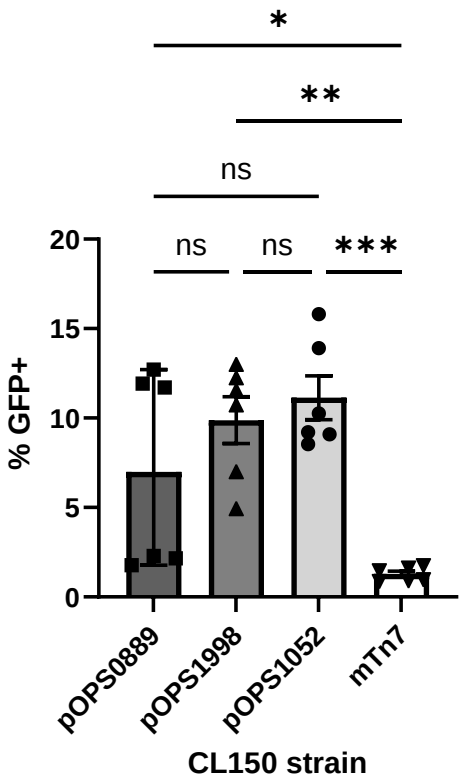


Figure 4.9 Mean percentage induction of CL150 SI biosensor strains on *RhiP* measured by flow-cytometry of mCherry marked strain recovered from the root-associated fraction of barley plants 7 days post inoculation. GFP fluorescence is measured relative to the 99th percentile of GFP induction from strains recovered from WT barley. (±SEM, n = 3, One way ANOVA with Tukey's multiple comparisons test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

The low copy RK2 biosensor pOPS1052 showed the greatest percentage of root colonising cells which responded to rhizopine induction (11.3%) whilst pBBR1 SI biosensors pOPS0889 and pOPS1998, with low expression transporters, showed slightly fewer average cells responding to rhizopine at 9.88% and 7.1% respectively, though neither decrease in mean population induction was significant from pOPS1052 ($p = 0.22, 0.92$) (*Figure 4.9*). The only biosensor which did show a significant decrease in percentage induction was mini-Tn7 biosensor pOPS1740 with pOPS0759 which had a mean population induction of 1.28% (*Figure 4.9*). This result suggests the dosage of SI transporters was too low or significant silencing of integrated transport gene expression occurred which abolished the strain's ability to respond to exogenous plant derived rhizopine. Integration of the SI transporters driven by the P*J23115* promoter is therefore ineffective for control of gene expression in response to plant host derived rhizopine. Development of stable integrated rhizopine biosensors will likely require further tuning of gene expression for use *in planta*.

4.3.3 Growth of *S. meliloti* Carrying SI Biosensors

Growth curves of CL150 strains carrying the previously discussed SI biosensors was also performed in a plate reader. Strains were grown in minimal media supplemented with succinate and ammonium in the presence or absence of 10 mM SI. Growth of RK2 SI biosensor strain carrying pOPS1052 was comparable to WT CL150 even with the addition of 10 μM SI. However, both pBBR1 high copy number plasmids pOPS0889 and pOPS1998 showed a significantly reduced growth rate when grown on media supplemented with SI (*Figure 4.10*). The reduced growth suggests the multi-copy expression of transporter genes in both plasmids was causing toxic accumulation of SI in these strains, and that the reduced expression from P*rpoD* was insufficient to alleviate this problem in *S. meliloti*.

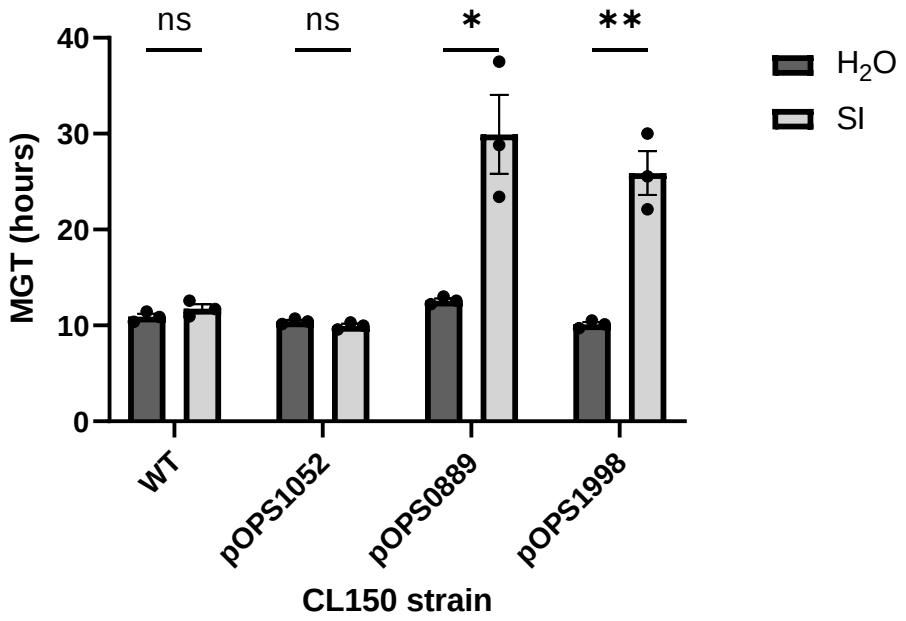


Figure 4.10 Mean generation time (MGT) of CL150 SI biosensor strains grown in minimal media supplemented with 10 mM nitrogen and 20 mM succinate at 28°C for 48 hours in the absence or presence of 10 µM SI. (± SEM, n =3, multiple independent two-tailed Student's T-test, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$).

4.4 Rhizopine Control of Symbiotic Nitrogen Fixation in *S. meliloti*

4.4.1 Construction of a *S. meliloti* NifA Controller

To control nitrogen fixation in response to exogenous SI in *S. meliloti*, the strategy of expressing *nifA* from a biosensor was developed for use in CL150. Ryu, MH *et al*, 2020 demonstrated that expression of *nifA* alone was sufficient to induce nitrogenase gene expression when the genomic sigma factor *rpoN* was left intact[99]. Based on the induction and growth curve results of SI biosensors in CL150, the RK2 vector pOPS1052 was selected to express *nifA*. The vector was digested with BamHI and *S. meliloti* native *nifA* and RBS was amplified with oxp4118/4935 and cloned via HiFi reaction into the vector to produce plasmid pOPS1791 (*Figure 4.11*). The sequence of pOPS1791 was confirmed by Sanger sequencing

with oxp5546/5547 before mobilising the plasmid from *E. coli* into *S. meliloti* CL150 Δ*nifA* to produce CL150 strain OPS2877.

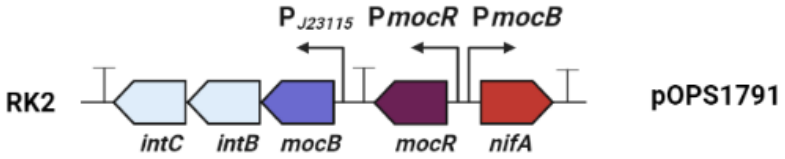


Figure 4.11 Plasmid construct (pOPS1791) for SI dependent expression of *S meliloti nifA*.

4.4.2 Control of *S. meliloti* NifA in *RhiP M. truncatula* Microcosms

To control nitrogen fixation in response to rhizopine in CL150, rhizopine producing T2 *Medicago truncatula* lines were obtained from Dr Ponraj Paramasivan which express the codon optimized *R. leguminosarum* inositol dehydrogenase gene *idhA,* and two transaminases *S. meliloti* L5-30 *mosB* and *Streptomyces griseus StsC* transaminase genes under the control of *Zea mays* ubiquitin1 promoter and *Oryza sativa* ubiquitin1 promoter, respectively. *RhiP Medicago* lines have been reported to produce 10ng mg^{-1} root dry weight SI (P. Paramasivan, unpublished)

Growth of WT *Medicago truncatula* using single plant microcosms was tested before scaling up to screening nitrogen fixation with the engineered rhizopine system. Growth of *M. truncatula* inoculated with symbiotic *S. meliloti* strains in microcosms provides a sterile environment with sufficient water and nutrients to ensure the sealed microcosm does not require opening during the assay, greatly reducing the possibility of contamination by other microbes of each plant replicate[281]. Furthermore, this method does not require a lot of space compared to potting experiments, allowing for inclusion of a greater number of plant replicates.

Microcosms were constructed from standard, round 100 mm diameter, 15 mm deep laboratory plates filled with ~70 mL FP agar nutrient media. The roots of *M. truncatula* were able to grow vertically down the agar surface whilst a notched opening in the top of the plate allows egress of the plant shoot to grow freely out of the microcosm (*Figure 4.12*). It has been reported that plants can be left to grow in this system undisturbed for up to 9 weeks[281]. At the end of the assay, plant roots can be lifted off the agar to study the nodulation phenotype and perform acetylene reduction to measure rates of nitrogen fixation.

Figure 4.12 Example of sterile microcosm for growth of *M. truncatula* in a 15 mm deep laboratory plate filled with ~70 mL FP agar nutrient media inoculated with WT *S. meliloti* CL150.

Seed pods of WT *M. truncatula* R108 or *RhiP* were cracked and the extracted seeds lightly scarified with sandpaper before sterilisation in 20% v/v NaClO solution for 2 minutes. Seeds were washed 10 times with 50 mL sterile ultrapure water and left to imbibe for 48 hours at 4°C before germinating the seeds on 1.2% agar H_2O square plate at 25°C. Seeds were incubated for 3 days until the roots were > 3 cm long to allow adhesion of the seedling to FP

agar media in the microcosms. Seedlings were placed in the microcosms with the emerging shoot aligned with the notched opening at the top of the plate. Plants were inoculated with 100 µL of overnight culture of *S. meliloti* diluted to OD 0.05 in sterile ultrapure water. The individual microcosms were placed in foil wrapped stacks in a lighted growth chamber at 22°C and 150 µmol m^{-2} s^{-1} light on a 16 hr light / 8 hr dark cycle.

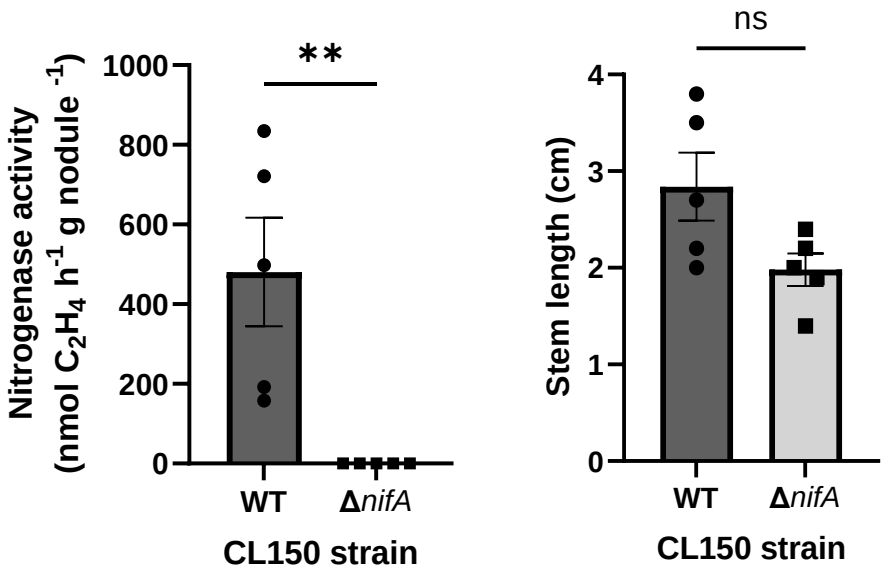


Figure 4.13 Mean nitrogenase activity (left) and stem length (right) of *M. truncatula* R108 grown in microcosms inoculated with WT CL150 or CL150 Δ*nifA* (OPS1507) measured 3 weeks post inoculation. (± SEM, n = 5, independent two-tailed Student's T-test ** $p \leq 0.01$).

M. truncatula microcosms inoculated with WT CL150 or CL150 Δ*nifA* (OPS1507) were grown for 3 weeks before measuring for symbiotic productivity via acetylene reduction assay and measurement of stem length. WT CL150 produced a mean nitrogenase activity of 480.7 (± SEM 136.2) nmol C_2H_4 h^{-1} g nodule $^{-1}$, with no fixation seen in plants inoculated with CL150 Δ*nifA* (*Figure 4.13*). The resulting stem lengths of fix- *M. truncatula* plants were on average 1.075 (± 0.455) cm smaller than CL150 WT fix+ plants however this difference was not significant at the 95% confidence level ($p = 0.056$) (*Figure 4.13*).

An assay of nitrogen fixation with both WT and *RhiP M. truncatula* plants grown in microcosms inoculated with either WT or SI *nifA* biosensor strain OPS2877 CL150 was performed. Fixation was only observed with plants inoculated with WT CL150, with no significant difference in the rate of fixation between WT and *RhiP M. truncatula* ($p = 0.67$) (*Figure 4.14*), no fixation was seen with SI *nifA* strain OPS2877 on either *RhiP* or WT *Medicago* plants. There was a large variation in the rates of nitrogen fixation seen with WT CL150, with a range of 2002.3 nmol C_2H_4 h^{-1} g nodule $^{-1}$, suggesting inconsistent rates of nitrogen fixation between different single plant replicates. The large range of activity measured could possibly be due to the reduced number of nodules per plant.

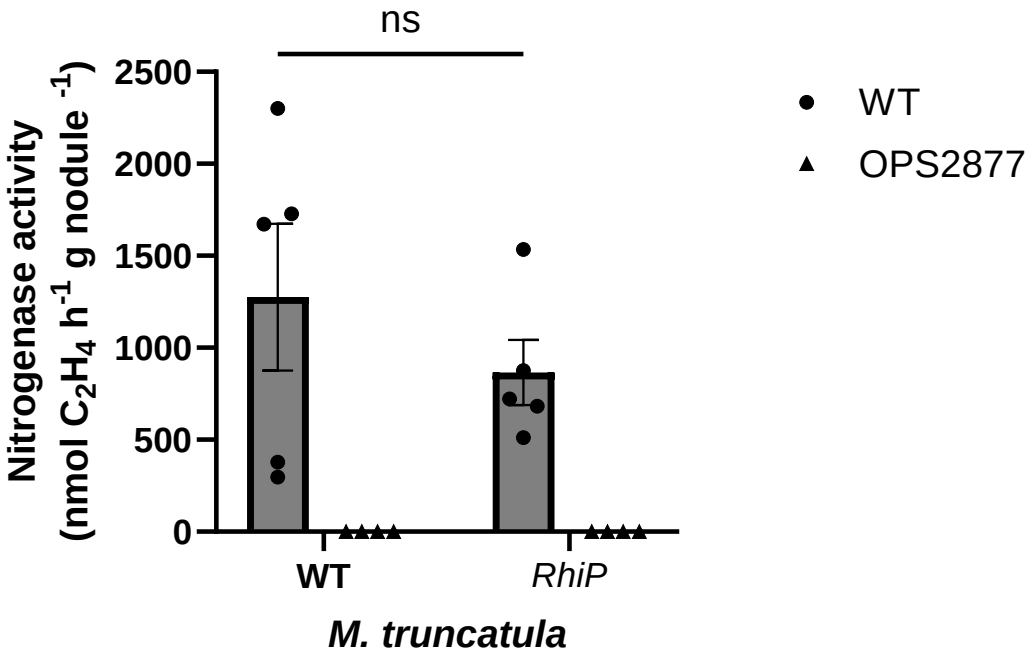


Figure 4.14 Mean nitrogenase activity of WT *M. truncatula* R108 and *RhiP M. truncatula* grown in microcosms inoculated with WT CL150 or CL150 Δ*nifA* OPS2877 carrying SI inducible *nifA* plasmid pOPS1791. Nitrogenase activity was measured 3 weeks post inoculation. (± SEM, n = 5, two-way ANOVA with multiple comparisons, ns $p > 0.05$).

4.4.3 Comparison of *M. truncatula* Growth Methods

Single plant microcosms for growth and analysis of *M. truncatula* were then compared to a single-pot experiment using fine sand as a growth media. Plastic pots were double stacked with a paper barrier sandwiched between the layers and filled with 50 mL fine-grade sand watered with 10 mL plant rooting solution supplemented with 1 mM KNO_3. Pots were autoclaved and individual pots planted with a germinated *M. truncatula* seedling and inoculated with 2 mL of washed OD_{600} 0.05 WT CL150 culture or left uninoculated. Pots were placed in trays to prevent cross-contamination between inoculants and grown in a lighted growth chamber at 22°C and 150 µmol m^{-2} s^{-1} light on a 16 hr light / 8 hr dark cycle. Pots were grown for 4 weeks with watering with 5 mL N-free rooting solution every 72 hours after the first week.

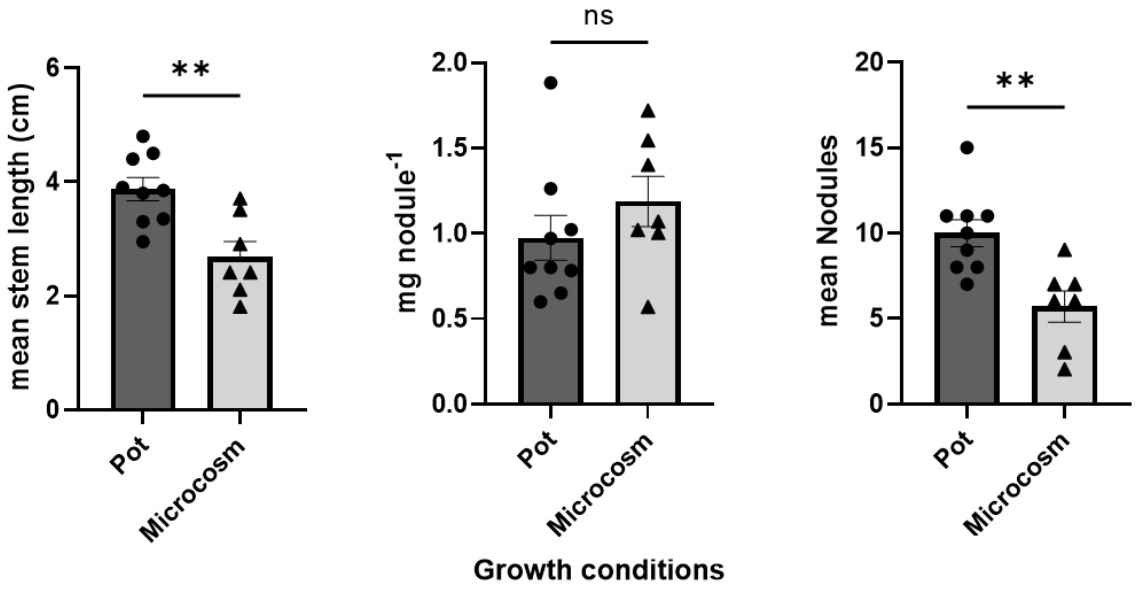


Figure 4.15 Mean stem length (left), mean weight per nodule (centre) and mean nodule number (right) of *M. truncatula* R108 grown in single sand pots or microcosms inoculated with WT CL150 measured 4 weeks post inoculation. (± SEM, n = 9, independent Student's T-test, ns $p > 0.05$, ** $p \leq 0.01$).

Comparison of plant growth and symbiotic phenotypes between WT *M. truncatula* grown in pots versus microcosms shows that the plants grown in pots with sand produce a more

robust symbiotic phenotype. *Medicago* grown in pots had on average a 1.18 (± 0.33) cm greater stem length and 4.29 (± 1.22) nodules more than plants grown in microcosms (n = 9, n = 7 respectively), there was no difference in the mean nodule mass between either experimental setup (*Figure 4.15*).

4.4.4 Control of *S. meliloti* NifA in Single Pot *RhiP M. truncatula*

As a greater stem length and nodule number implies a greater rate of nitrogen fixation, a repeat of the nitrogen fixation assay with *RhiP* plants inoculated with SI *nifA* biosensor strain OPS2877 grown in sand pots was performed on plant three weeks post inoculation. However, again fixation was only seen in plants inoculated with WT CL150, with no difference in rates of nitrogen fixation between WT or *RhiP* lines of *Medicago* ($p = 0.66$, $n = 9$). No fixation was seen with acetylene reductions performed with *RhiP Medicago* inoculated with OPS2877 (*Figure 4.16*). Measurement of plant stem lengths showed a significant decrease in the mean length between fix+ WT inoculated *RhiP* plants and fix- OPS2877 *RhiP* plants (-0.76 ± 0.34 cm, $p = 0.047$, $n = 9$), suggesting fixation did not occur at any point during growth (*Figure 4.16*). OPS2877 was able to nodulate *RhiP M. truncatula* but produced on average 4.56 (± 0.94) more nodules than WT CL150 inoculant on *RhiP* plants (*Figure 4.16*). Furthermore, inspection of OPS2877 *M. truncatula* nodules showed small white nodules compared to healthy pink nodules containing leghaemoglobin produced by WT CL150 inoculum. White nodules indicate a poor symbiosis and lack of induction of *nif* genes by SI in this engineered system.

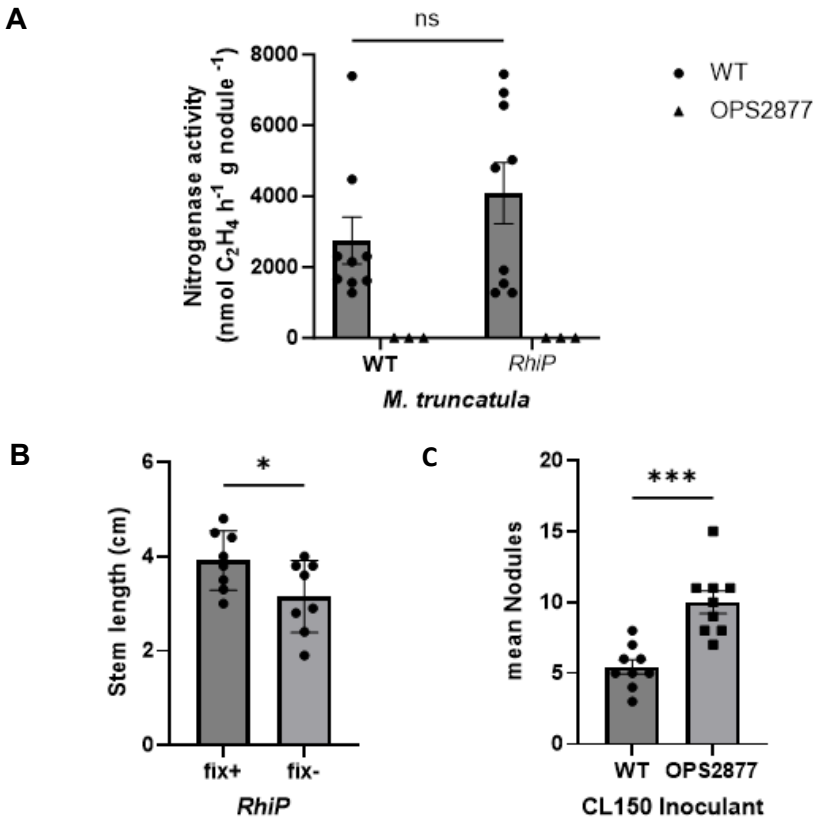


Figure 4.16 A Mean nitrogenase activity of *M. truncatula* R108 or *RhiP* inoculated with WT or OPS2877 CL150, plants were grown in sand filled pots and measure 4 weeks post inoculation (± SEM, n = 9, two-way ANOVA, ns $p > 0.05$). **B** mean stem length of *RhiP M. truncatula*, fix+ plants were inoculated with WT CL150, fix- plants were inoculated with OPS2877 (± SEM, n = 9, independent Student's T-test * $p \leq 0.05$). **C** mean number of nodules per plant of *RhiP M. truncatula* inoculated with WT CL150 or OPS2877 (± SEM, n = 9, independent Student's T-test, ** $p \leq 0.001$).

4.4.5 Flow Cytometry of *S. meliloti* SI Biosensors on *RhiP M. truncatula* and Barley

To measure induction of SI biosensors in the nodules of *RhiP M. truncatula,* flow cytometry was performed on CL150 mCherry marked strain carrying the RK2 SI biosensor pOP1052 (OPS3509). Plants were grown in sand using the method previously discussed and nodules extracted and crushed three weeks post inoculation. Nodule extract was resuspended in 50 µL PBS and analysed by autosampling flow cytometry. Cells were excited for GFP and mCherry fluorescence with 488 and 561 nm lasers, respectively. Bacterial singlets with mCherry fluorescence above 5,000 a.u were considered as mCherry+ bacteria for analysis. Within the mCherry+ bacterial population, cells exhibiting GFP fluorescence above the mean 99th percentile determined for bacterial population isolated from WT *Medicago* nodules were classified as GFP+ cells responding to SI. Induction of CL150 SI in *Medicago* nodules was compared to the same strain isolated from the roots of *RhiP* barley 9-days post inoculation, with cells extracted from the root using the method previously discussed.

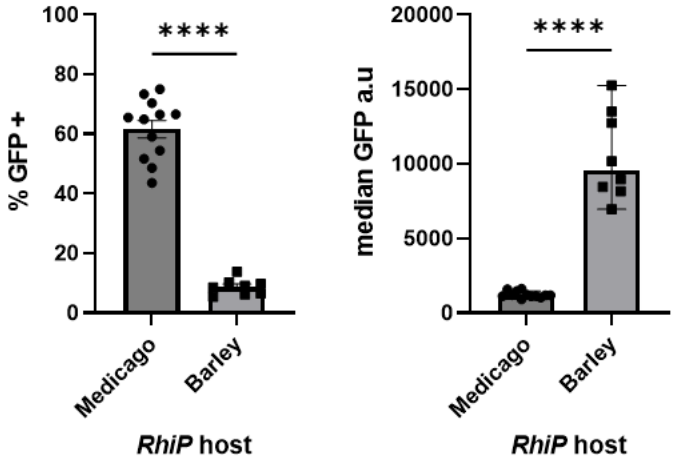


Figure 4.17 Left Percentage induction of mCherry marked CL150 carrying the RK2 SI biosensor pOP1052 (OPS3509) recovered from nodules of *RhiP M. truncatula* and root associated fraction of *RhiP* barley. **Right** Median GFP of induced fraction of OPS3509 on *RhiP M. truncatula* and *RhiP* barley (± SEM, n = 12 and 8 respectively, independent Student's T-test, **** $p \leq 0.0001$).

CL150 extracted from *RhiP Medicago* nodules exhibited a mean induction of 60.66% of the bacterial population which was 52.93 (± 3.68) greater than induction seen in root associated populations of the same bacteria on the roots of *RhiP* barley (*Figure 4.17*, mean 8.73%, $p = 0.002$, $n = 12, 8$ respectively). However, there was a significant difference in GFP induction in the induced bacterial populations between *Medicago* and barley, with root associated barley populations exhibiting 10-fold greater median GFP induction relative to induced *Medicago* nodule populations ($p \leq 0.0001$, *Figure 4.17*).

4.4.6 Imaging of *S. meliloti* SI Biosensors on *RhiP M. truncatula*

Induction of CL150 in the nodules of *Medicago* was further visualised by constructing an mCherry SI biosensor for stereo microscope imaging of bacterial induction in *RhiP* nodules. An mCherry biosensor was required due to the constitutive expression of eGFP from *RhiP Medicago* lines. The RBS-mCherry-DT16 expression module was isolated from pOPS1531 using primers oxp5822/5823 and cloned via HiFi reaction into pOPS1889 digested with BamHI to produce plasmid pOPS1987. The resulting plasmid was conjugated into CL150 (OPS3259) and inoculated on *RhiP Medicago.* Plants were grown for 3 weeks post inoculation before uprooting and visualising nodule fluorescence using a Leica stereo microscope. As expected, GFP fluorescence was visible throughout the nodules in *RhiP* lines but not WT R108 *Medicago* (*Figure 4.18*). However, the resulting mCherry induction in response to SI production by *RhiP* lines was only weakly visible in small pockets of nodules, suggesting poor induction and weak expression of the SI mCherry biosensor in CL150 bacterial populations (*Figure 4.18*).

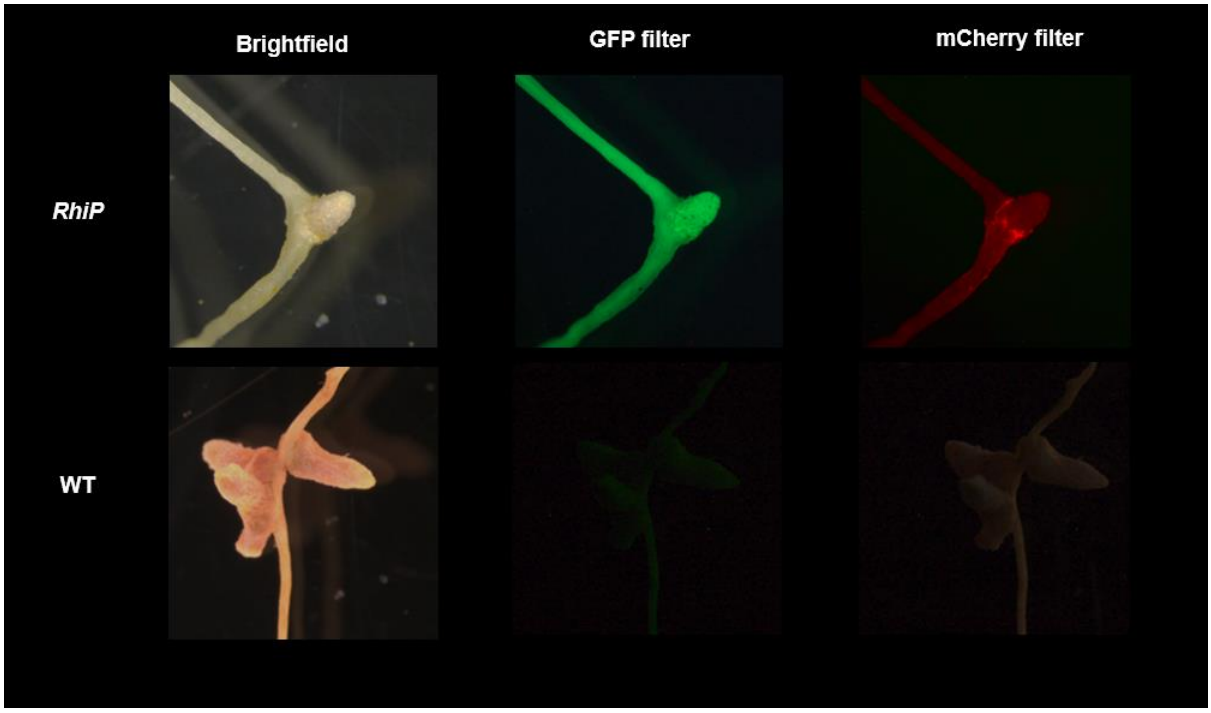


Figure 4.18 Stereo microscopy images of SI mCherry induction in CL150 strain OPS3259 inoculated on WT and *RhiP* lines of *M. truncatula.* Images taken using a Leica fluorescence stereomicroscope with GFP and mCherry filter.

4.5 Discussion

Using the refactored cluster *Klebsiella oxytoca* cluster v2.1 with a IPTG inducible T7 RNA polymerase controller, free living nitrogen fixation was achieved in *E. coli* and *S. meliloti* CL150. However, the rate of nitrogenase activity was 20-fold lower in *S. meliloti* compared to *E. coli*. No activity was seen in either strain with refactored cluster v3.2. Subsequent sequencing of refactored cluster v3.2 showed five SNPs in coding genes, with a transition mutation in *nifB* resulting in an amino acid substitution at residue 276 from aspartic acid to glycine. These mutations were not present in the sequencing of cluster v2.1. Furthermore, sequencing showed 2 SNPs present in all P2 promoters encoded on the plasmid which would have likely altered the expression ratios of *nif* operons which has been shown to be important

for optimal function of nitrogenase in heterologous hosts[99]. Growth curves of CL150 carrying refactored cluster v3.2 showed markedly decreased growth relative to the WT, suggesting the cluster placed a large metabolic burden on cells. The metabolic stress likely led to a higher rate of mutagenesis, resulting in inactivity of *nif* genes. The refactored *nif* cluster v2.1 achieved significantly increased activity of nitrogenase in *E. coli* compared to the transfer of cloned *k. oxytoca* native *nif* genes, due to combinatorial optimisation of gene modules[204,205]. However, no activity was seen in *P. protogens* Pf-5 or *Rhizobium sp.* IRBG74. Testing this cluster under our experimental conditions, the *nif* cluster achieved comparable levels of nitrogen fixation in *E. coli,* but surprisingly also produced small levels of fixation in CL150, though the cluster was still inactive in Pf-5 and IRBG74.

The activity of *K. oxytoca* refactored *nif* cluster v3.2 in *Rhizobium sp* IRBG74 was the first demonstration of N_2 fixation with gammaproteobacterial *nif* genes in an alphaproteobacterium, this was achieved through reverting to a natural operonic structure with optimised genetic components tested in IRBG74. However, this improvement only achieved low rates of nitrogen fixation, with greater nitrogenase activity produced in *Rhizobium* by expressing the native *nif* genes from *R. sphaeroides*[99]. It was also reported that the native *K. oxytoca nif* cluster performs similarly when transferred to different species, but the refactored cluster v2.1 that uses codon optimization and disrupts natural operon structure produced varying levels of expression between bacterial genera[99]. Activity was restored by recovering the native operon structure to maintain relative gene synthesis rates in cluster v3.2[99]. Therefore, the expression of genes in cluster v2.1 in *S. meliloti* must have been sufficient to produce a functional nitrogenase and associated accessory components. Even if the v3.2 cluster had been functional, this may not have been the case in *S. meliloti* and therefore demonstrates the need for iterative testing of *nif* cluster functionality followed by gene reorganisation using a toolbox of synthetic biology parts for optimal function of *nif* clusters in new bacterial hosts.

This study went on to demonstrate the transfer of 12 *nif* gene clusters between 15 bacterial species for nitrogen fixation[99]. The results of this study indicate that the expression and activity of refactored *nif* genes from one species is dependent upon the genotype of the intended expression host. However, this can be overcome by targeted engineering of the host genome, for example, co-expression of *P. polymyxa* electron transporter genes *pfoAB-fldA* and the *K. oxytoca* nitrogenase Fe-S cluster assembly genes *nifSU* alongside the *nif* operon of *P. polymyxa* WLY78 in *E. coli* improved nitrogenase activity by 50%[203]. The quantification of operon expression levels and translational parts, such as RBSs, via ribosome profiling and RNA sequencing would be required to further elucidate the problems associated with the engineered *nif* clusters and to optimise expression in CL150. With only very low rates of nitrogenase activity seen with v2.1 cluster in CL150, this is not an effective strategy to control nitrogen fixation in a symbiotic strain.

Balancing the requirements of optimal stoichiometric gene expression with removal of native gene regulation remains a major challenge in engineering nitrogen fixation for agricultural inoculants. So far, combinatorial assembly and reiterative cycles of optimised gene expression for a particular host has been the most effective strategy to facilitate the transfer of *nif* genes between bacterial hosts. Another strategy has been demonstrated by utilising the posttranslational protein-splicing strategy derived from RNA viruses to minimise the gene numbers required for a functional nitrogenase. The translation of genes into polyproteins reduces the number of translational units and maintains stoichiometric expression, thus simplifying the engineering approach of complex biological systems such as BNF. *K. oxytoca* *nif* gene operons were fused to produce one large gene, which by expressing *nif* genes as a polyprotein with cleavage sites for Tobacco Etch Virus protease, maintained stoichiometric expression of the nitrogenase system. Through iterative design this led to an engineered system with only five genes, which when processed by the protease, enabled synthesis of an active

nitrogenase complex with 72% activity relative to native *K. oxytoca* and allowed diazotrophic growth of *E. coli*[282]. This strategy provides a promising alternative for effective balanced expression of nitrogenase components whilst simultaneously reducing the number of genes required to engineer BNF in foreign hosts[282]. Despite the advances achieved by these engineering methods for the transfer of *nif* genes, fully developing engineered strains as cereal inoculants for effective BNF will require further engineering of metabolism for optimal utilisation of carbon sources and delivery of fixed nitrogen, as well as introduction of genes for effective electron transfer to the nitrogenase complex. An endophyte would further require engineering of oxygen protection mechanisms seen in free living diazotrophs such as *A vinelandii* to facilitate microaerobic nitrogenase activity. Therefore, the choice of bacterial chassis alongside the transfer *nif* gene clusters would have to be considered carefully.

To achieve control of nitrogen fixation in *S. meliloti,* we resorted to the use of *nifA* expression which proved successful in *A. caulinodans*. As expression would be controlled by an exogenous rhizopine signal, a series of engineered SI biosensors were tested in CL150 for optimal stable gene expression from *PmocB* in CL150. The functionality of SI biosensors was previously tested in a range of rhizobial alpha-proteobacteria, *S. meliloti* strains exhibited a 10-fold greater induction in response to SI compared to *A. caulinodans* when carrying the pBBR1 biosensor pOPS0889. The increase is likely due to the genetic parts for rhizopine induction being taken from *S. meliloti* L5-30, leading to optimal activity of the sensor in *S. meliloti* strains due to compatibility. The induction of pOPS0889 in *A. caulinodans* was also found to negatively affect the growth of strains, particularly in the presence of 10 μM SI in the growth medium. This growth defect was also found to be the case in *S. meliloti* CL150, though growth was not significantly affected without the presence of SI.

Growth was likely affected by the addition of SI due to toxic accumulation of SI within the cell by rhizopine transporters IntBC. To overcome this, three different rhizopine biosensor

designs were screened for response to SI, impact of growth, and stability of induction in CL150. To reduce the dosage of SI transporters in CL150, integration of the SI biosensor by mini-Tn7 chromosomal integration was tested. Induction could not be seen by microplate assay in response to SI with the integrated biosensor, significant *gfp* induction required complementation with the *mocR*-P*mocB::gfp* plasmid pOPS0759 which produced a comparable response to 10 μM SI to the pBBR1 plasmid pOPS0889. The inability of the integrated biosensor to show SI-inducible *gfp* expression could be due to insufficient levels of the MocR or MocB proteins or the associated reduced dosage of *gfp*. Furthermore, integration of SI transporters and perception genes led to comparable growth to the wild-type control CL150 in the presence and absence of 10 μM SI. However, analysis of induction after 9-days post inoculation on *RhiP* lines of barley showed that induction was severely reduced and was not significantly different from the background induction measured on WT barley. The lack of induction with the integrated biosensor suggests that the dosage of transporters was too low to transport the reduced concentrations of rhizopine exuded from the roots of SI barley, or that single gene copies of transporters were susceptible to mutagenesis over the course of the *in planta* assay rendering the strain unable to respond to rhizopine.

A second strategy to alleviate SI toxicity tested was to reduce the expression of transporter genes whilst maintaining high copy number of SI perception genes. The *S. meliloti* promoter P*rpoD* was identified as having weaker expression than P*J23115* and could therefore be used to drive lower levels of *intBC* expression on a high copy pBBR1 replicon SI biosensor (pOPS1998). Induction of P*mocB*::*gfp* in plasmid pOPS1998 produced a greater dynamic range of expression across the range of SI concentrations tested than the previous pBBR1 plasmid pOPS0889 (76.8-fold versus 44-fold induction). However, SI perception by pOPS1998 was less sensitive relative to pOPS0889 induction across all concentrations, presumably due to the reduced expression of transporters. Furthermore, growth curve analysis of CL150 carrying

pOPS1998 still showed a growth defective phenotype, as seen with the other pBBR1 SI biosensor pOPS0889, with the MGT doubling in in the presence of 10 μM SI; suggesting that the decrease in *intBC* expression was insufficient to relieve the metabolic burden or toxicity imparted on the cell due to the high-copy number of vector.

Analysis of stability of induction of both pBBR1 plasmids in CL150 showed a slightly greater mean percentage induction with pOPS1998 compared to pOPS0889 on the roots of *RhiP* barley, though the difference was not statistically significant (mean difference = 2.79, p = 0.53). Both strains showed roughly double the percentage induction compared to the relative activity reported by *A. caulinodans* SI biosensor strains on roots of *RhiP* barley[283], reflecting the relative increase in activity of SI perception genes in their native genomic background. Finally, a low-copy biosensor was tested with SI transport and perception genes cloned from the original pOPS0889 plasmid onto a RK2 origin plasmid vector which also encodes PAR genes for faithful plasmid segregation at cell division[284]. This plasmid still retained the ability to perceive SI, albeit with six-fold lower maximal induction relative to pBBR1 plasmid pOPS0889. Furthermore, RK2 plasmid pOPS1052 exhibited wild-type growth characteristics whether in the presence or absence of SI, suggesting that reducing the dosage of all SI biosensor genetic parts alleviates toxicity. *S. meliloti* carrying pOPS1052 resulted in the highest mean percentage (11.3%) of cells showing induction on the roots of *RhiP* barley, though this was not statistically significant from pOPS0889 induction ($p = 0.21$). Using this analysis of SI biosensors, it was decided that an RK2 SI biosensor to express *nifA* would be the most effective strategy to control nitrogen fixation in *S. meliloti* on *RhiP Medicago.*

An SI *nifA* biosensor using pOPS1052 as the vector backbone was constructed for induction in the nodule environment. This controller was initially tested in *Medicago* grown in microcosms of FP agar in sealed petri dish plates. Microcosms functioned well for WT lines of *Medicago* which showed good rates of nitrogen fixation per plant, albeit with a large

variance in rates. However, when tested with T2 lines of *RhiP Medicago,* no fixation was seen with CL150 carrying the RK2 SI *nifA* biosensor, which resulted in a significant decrease in the mean stem length relative to plants inoculated with WT CL150. Growth of *Medicago* in microcosms also produced fewer nodules per plant compared to pot-based growth methods which likely reduced the overall rate of nitrogen fixation and other phenotypic differences. Furthermore, difficulty came from using the microcosms with T2 lines as they produced shorter roots than the WT after the same period of germination, which prevented many from adhering to the nutrient agar and failing to mature. Therefore, the growth method was changed to individual sand pots with a regular watering schedule with N-free rooting media. This method produced larger plants with more nodules, but still failed to show any fixation with SI producing lines and OPS3259 CL150. Analysis of plants by flow cytometry showed much higher induction of cells relative to barley but overall lower median GFP fluorescence. This result indicates that cells were more exposed to SI in the nodule environment relative to the root surface, but that the overall concentration of SI was lower as it induced a weaker response from P*mocB*. Reduced induction was evident when visualising mCherry induction in response to SI in *RhiP* nodules, where pockets of weak fluorescence were visible throughout nodules. Weak induction suggests the sensing of rhizopine was not stable over the three-week growth period of the plants or that low levels of SI were present in the nodules leading to weak induction relative to CL150 on the root surface of T2 *RhiP H. vulgare*.

It could therefore be possible that due to the inefficiency of induction of SI *nifA* CL150 conditional sanctioning could have occurred. Sanctioning is seen with ineffective rhizobial partners, whereby nodules show restricted development due to the plant hosts providing fewer resources by cutting off the nodule supply of carbon, oxygen, or other nutrients[69,285-287]. Host sanctioning could have occurred in the engineered rhizopine system, with low concentrations of SI preventing adequate induction of *nifA* for *nif* gene expression and fixation. Furthermore,

SI may not be present in the nodule environment despite being produced in the root, which may explain the lack of rhizopine induced mCherry induction throughout the nodule in microscopy images of *RhiP* plants inoculated with OPS3259. To improve engineered synthetic symbiosis, it will likely be required to further engineer the production SI in the plant host for increased bacterial induction, alongside tuning rhizopine responsive genes in the corresponding bacterial partner so that there is less of a metabolic burden leading to improved stability. It may also be required to find alternative orthogonal signals which either occur naturally as plant species specific signals, or a completely synthetic signal, as these may provide orthogonal induction of bacteria with greater sensitivity and stability of gene expression for nitrogen fixation. Metabolomics studies performed on plant root exudates have shown that some plants produce unique secondary metabolites which may be used as plant-specific signal for control of bacterial gene expression in the rhizosphere[224].

Chapter 5

Control of PGP and N_2 Fixation in Diverse Rhizobacteria

5.1 Engineering IAA Production for PGP Overview

Use of plant growth promoting bacteria as cereal inoculants goes beyond just nitrogen fixation. Recent studies have elucidated many mechanisms beyond BNF in rhizobacteria which lead to plant growth promotion (PGP). The engineering and transfer of PGP traits to a bacterial chassis is an exciting prospect for the continued development of effective bacterial inoculants for agricultural use. Similarly to BNF, these PGP traits can be controlled by plant host specific signalling to establish tightly regulated and specific PGP by plant colonising bacteria. Several mechanisms of PGP by bacteria have already been commercialised, however their field performance is often inconsistent due to lack of host-specificity, suboptimal colonisation of the rhizosphere due to competition by existing microbes, and lastly by native genetic regulation by the host bacterium to repress PGP traits under disadvantageous conditions to conserve energy[165]. However, many mechanisms of PGP including N_2 fixation, phytohormone production, phosphate solubilisation, rhizoremediation, and biocontrol of pathogens, have been studied enough to be conducive to genetic engineering in a bacterial inoculant under synthetic host-specific signalling as demonstrated by the engineered rhizopine system. Establishing control of PGP in bacterial chassis will allow us to optimise the specificity, effectiveness, and ecology of an engineered PGP strain to overcome the limitations of natural PGP.

One enticing mechanisms of PGP is the production of plant phytohormones. Study of the rhizosphere and plant development has shown the important role in which auxins, such as indole-3-acetic acid (IAA), have as natural phytohormones for PGP. Auxins such as IAA have a wide impact on plant development, from cell differentiation and division, lateral root formation, and improved host-plant tolerance to abiotic stress[172]. In bacteria, auxin can improve the ability of rhizobacteria to colonise roots and has been shown to affect gene expression

suggesting its use as a signalling molecule[171]. Many rhizobacteria have been shown to produce auxin, with the best studied examples being the phytopathogenic bacteria *Agrobacterium* and *Pseudomonas savastanoi* which cause host tumours and galls due to IAA induced plant growth.

However, IAA production is also documented in PGP bacteria such as *A. brasilense* which regulated plant root development and growth. Synthesis of IAA has been reported in many bacteria isolated from the rhizosphere, although production varies between species[171]. The precursor for the synthesis of IAA is the amino acid tryptophan, though from this starting point at least five different biosynthesis pathways have been documented, though not all the intermediates and enzymes required have been characterised. The indole-3-acetamide (IAM) pathway is found in pathogens such as *P. savastanoi* and produces high amount of IAA which contributes to the strain's pathogenicity[172]. The pathway consists of two steps, the first converting tryptophan to IAM by tryptophan monooxygenase (encoded by *iaaM*), and secondly IAM is hydrolysed to IAA and ammonia by a hydrolase (encoded by *iaaH*) (*Figure 5.1*)[119]. This pathway differs from the main auxin biosynthesis pathway in plants, the indole-3-pyruvate (IPA) pathway, which converts tryptophan to IAA in three steps. Firstly, via transamination to an IPA intermediate, which is decarboxylated to indole-3-acetaldehyde (IAAld), and finally oxidised to IAA by a dehydrogenase[170]. The IPA pathway is present in PGP bacteria such as *A. brasilense* and *Bradyrhizobium japonicum*. IAA acts as a signalling molecule and positive regulator of the dehydrogenase gene *ipdC* in the IPA pathway of *A. brasilense*, where IAA production is increased via positive feedback during culture growth[118].

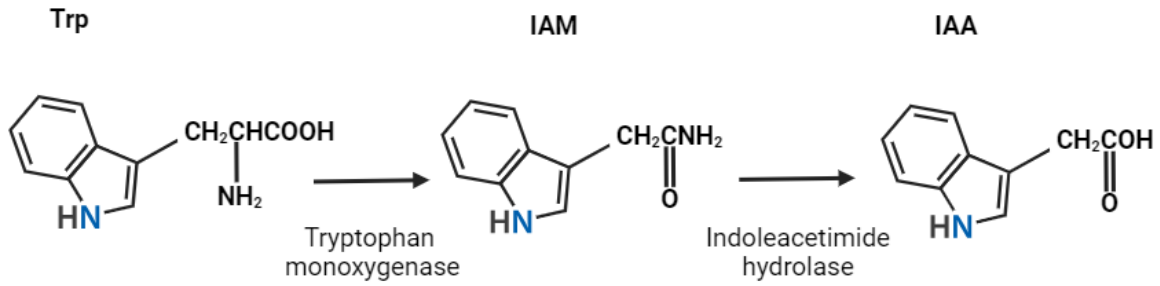


Figure 5.1 Bacterial indole acetamide pathway found in bacteria such as *Pseudomonas savastanoi.*

Auxin is involved in many of the processes required for nodule formation in legumes including nodule initiation and differentiation via auxin accumulation[119]. Therefore, many rhizobia have been found to produce IAA which can alter the phytohormone balance inside the plant roots to facilitate symbiosis. Furthermore, a *S. meliloti* strain overexpressing the IAM biosynthetic pathway showed increased tolerance to several stresses, and *M. truncatula* plants inoculated the IAA overexpressing strain had a higher auxin content in nodules and roots and were better resistant to salt stress and phosphorus deficiency[180,181]. Endophytic strains such as *A. brasilense* and *K. pneumoniae* have also been studied for the effect of their production of auxins on the growth and development of rice roots[116,117]. Culture supernatants from these strains grown in media supplemented with tryptophan was applied to the roots of hydroponically grown rice and was found to increase root elongation, lateral root and root hair growth, as well as root surface area and dry weight, whilst high IAA supernatant concentrations lead to the formation of nodule like tumours[117]. *Azospirllum* has also been studied for its ability to improve rhizobia-legume symbiosis due to its phytohormone production through co-inoculation[116]. Soybean growth promotion was measured through co-inoculation with the symbiotic partner strain *Bradyrhizobium japonicum* and *A. brasilense* and was found to increase total plant and root length, aerial and root dry weight, nodule number and weight, and an increase in the symbiosis established with *B. japonicum* after foliar co-inoculation with IAA

producing *A. brasilense* compared to a knockout IAA- mutant strain[118]. These studies demonstrate that the presence of microorganism producing IAA as part of the colonisation process contributes to positive effects on plant growth and establishment of symbiosis. Therefore, the engineering of IAA production into a rhizobacterial inoculant could improve PGP for host crop species, or be used as a plant-specific signal to induce further symbiosis specific genes in an engineered system. The indole-3-acetamide biosynthesis pathway has been successfully transferred from *P. savastanoi* for IAA production in *Curavidus pinatubonensis* which was able to increase lateral root number, root length, and plant fresh weight when inoculated on *Arabidopsis*[182]. Therefore, the expression of the IAA pathway under control of rhizopine in an engineered bacterial chassis could lead to further PGP alongside nitrogen fixation.

5.2 Engineering Relay Signalling Overview

A facet associated with rhizopine signalling which needs to be addressed is the functionality of SI induction genes across bacterial taxa. The host range of rhizopine signalling was tested using the induction of *gfp* expression from SI plasmid pOPS0889 in a range of alpha-, beta-, and gamma-proteobacteria. It was found that SI induction of gene expression was limited to a small number of rhizobial alpha-proteobacteria species[249] (*Figure 5.2*). Further analysis of SI biosensor induction demonstrated SI transport was possible in non-alphaproteobacterial strains but *moc* promoters and possibly MocR itself were incompatible with host transcriptional machinery in these heterologous strains[248]. Since MocR is a positive regulator, it is possible it has evolved interactions with the RNA polymerase required for activation that are exclusive to alphaproteobacteria. Despite the effectiveness of SI signalling in rhizobia, there are diverse bacterial species capable of N_2 fixation and other PGP mechanisms which would be ideal targets to be brought under plant-host specific control. Therefore, the biosynthesis of a secondary signal molecule could be placed under SI control in

a cognate rhizobial partner such as *S. meliloti* and used to relay host-specific control to further rhizobacteria carrying a second cognate inducible gene circuit for expression of PGP genes.

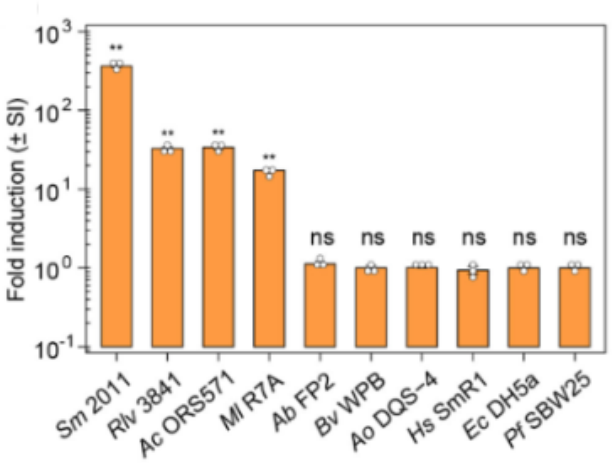


Figure 5.2 Rhizopine inducible *gfp* expression as fold-induction normalised to uninduced cells, tested in a range of bacteria (*Sm*, *Sinorhizobium meliloti*; *Rlv*, *Rhizobium leguminosarum* bv. *viciae*; *Ac*, *Azorhizobium caulinodans*; *Ml*, *Mesorhizobium loti*; *Ab*, *Azospirillum brasilense*; *Bv*, *Burkholderia vietnamienses*; *Ao*, *Azoarcus olearius*; *Hs*, *Herbaspirillum seropedicae*; *Ec*, *E. coli*; *Pf*, *Pseudomonas stuzeri*) carrying the rhizopine biosensor plasmid pOPS0889 grown with 10 µM SI supplemented into the growth media. Adapted from Haskett *et al,* 2022[249].

For example, acyl-homoserine lactone (AHL) biosynthesis has been used to activate quorum-sensing genes that respond to these signals to control PGP traits[165]. Quorum sensing via AHLs has also been shown to allow complex interactions between bacteria of different species are usually involved in adaptive changes in physiology of the bacterial population, such as antibiotic production and transfer of mobile genetic elements. AHLs act as autoinducers (AI) which are constitutively synthesised at a low rate and excreted from the bacterial cell. When the concentration of these signal molecules reaches a defined threshold (quorum), either due to an increased cell density or habitat conditions, a receptor protein (R-protein) can bind the AHL

and induce the transcription of quorum sensing-regulated genes, but also AI biosynthesis genes which amplify the response[288,289].

AHL production by plant-associated bacteria is common, with isolates belonging to the generas *Agrobacterium*, *Rhizobium*, *Sinorhizobium*, *Pantoea*, *Erwinia*, *Pseudomonas* and *Xanthomonas* producing AHLs. Different species can produce the same AHLs or AHLs with slight variations in structures, such as length of acyl chains, leading to different properties. AHLs produced by rhizosphere bacteria can be key for optimal host colonisation of bacteria and their ability to form biofilms[54,265]. Bacterial AHLs can also induce systemic plant defences, whereby a strain of *Serratia liquefaciens* inoculated on tomato roots produced AHLs which conferred resistance to fungal pathogens to the host plant[197]. Alongside defence and stress related proteins, bacterial AHLs have also been shown to activate plant genes associated with protein and flavonoid metabolism, and hormone production, such as the activation of auxin specific promoters[290,291]. Due to the versatility of AHLs, they have also been used in engineered systems, such as to control IAA production in *Curavidus pinatubonensis*[182], as well as in controllers for nitrogenase activity in *E. coli* [99]. Although, most AHL sensors are specific to their cognate AHL signal, the ubiquity of these signals has led to the evolution of broad-range receptors capable of promiscuous responses. Diverse receptors expands the functionality of specific AHLs to interspecies interactions which regulate competition or cooperation in complex microbial communities[292]. Despite the very similar structures of natural AHL signals, comprised of a homoserine lactone core with an acyl tail, the acyl tails can vary in length from 4 to 20 carbons and in modifications to carbons which can be unsaturated or have a hydroxy or oxo modification, giving some specificity to receptor interactions[293] (*Figure 5.3*). Roughly 20 different naturally produced AHLs have been characterised from hundreds of quorum-sensing organisms, suggesting some degeneracy in signal responses and trans-species crosstalk[289]. However, AHL analogues have been produced using engineered AHL synthases and cognate

receptors which preserve specificity and could prevent interference from native systems and promiscuous cross-talk[293].

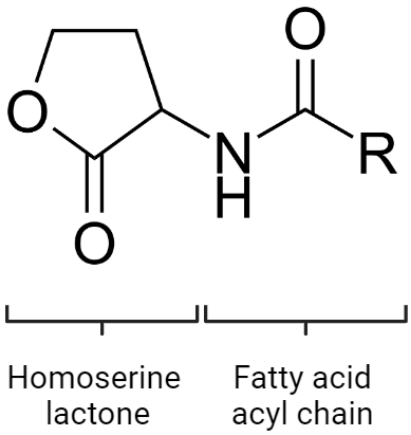


Figure 5.3 Structure of Acyl-homoserine lactones (AHLs) produced by bacteria, modifications of the acyl chain, denoted by R, confer specificity.

A further use of a rhizopine relay signal in PGP could be to control plant pathogens, which would also help reduce the widespread use of pesticides in agriculture. Pesticides have been a significant contributor to agricultural productivity, however, many contain organic pollutants which can persist in the environment and may accumulate in organisms within the ecosystem to potentially toxic levels, their use also disrupts the soil microbiome and can reduce the efficiency of nitrogen fixing symbiosis with host plants[161,188]. Plant colonising *Pseudomonas* species have already been studied in this particular aspect due to their ability to produce a variety of broad-spectrum antifungal molecules such as 2,4-diacetylphloroglucinol (2,4-DAPG), phenazines, pyrrolnitrin, and pyoluteorin[156,187,190,192,208,294]. One well researched molecule which could be effective is the antifungal 2,4-DAPG which is produced by the biosynthetic gene cluster *phlDACB* from *P. protegens Pf-5* and is effective in pathogen inhibition as well as induction of host plant defences[192]. Biosynthesis of 2,4-DAPG is carried out by the condensation of three molecules of malonyl-CoA to produce phloroglucinol by a

polyketide synthase encoded by the *PhlD* gene. Phloroglucinol is further modified by the transfer of acetyl groups at the third and fourth position by an acetyltransferase encoded by *phlACB* leading to 2,4-DAPG (*Figure 5.4*)[295].

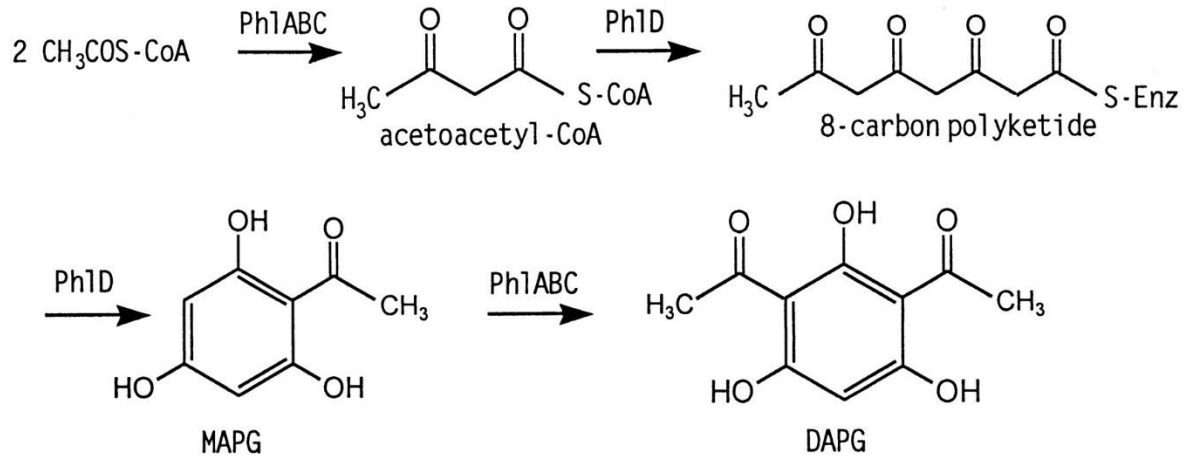


Figure 5.4 2,4-diacetylphloroglucinol pathway and the biosynthesis genes involved from the species *Pseudomonas fluorescens.* Adapted from Bangera et al, 1999 [296].

Pseudomonas strains conferred the ability to synthesise 2,4-DAPG through the transfer of *Phl* genes have been shown to enhance antagonism towards *Pythium ultimum* and *Rhizoctonia solani* which causes plant damping-off disease[192]. Endophytic colonization by *Phl* engineered recombinant *Pseudomonas* strains conferred plant protection and thereby the improved the performance of rice, sorghum, wheat, and sugar beet challenged with fungal pathogens[297,298]. By bringing the biosynthesis of a 2,4-DAPG secondary metabolite under SI control in an engineered rhizobial host, an engineered strain could provide biocontrol of pathogens alongside nitrogen fixation for PGP. Furthermore, the SI signal could be relayed via 2,4-DAPG or AHL signalling molecules to more diverse PGP rhizobacteria, carrying a second

cognate derepressible promoter system controlled by the PhlF regulatory repressor protein found in the *phl* operon, or the LuxR AHL regulator (*Figure 5.5*).

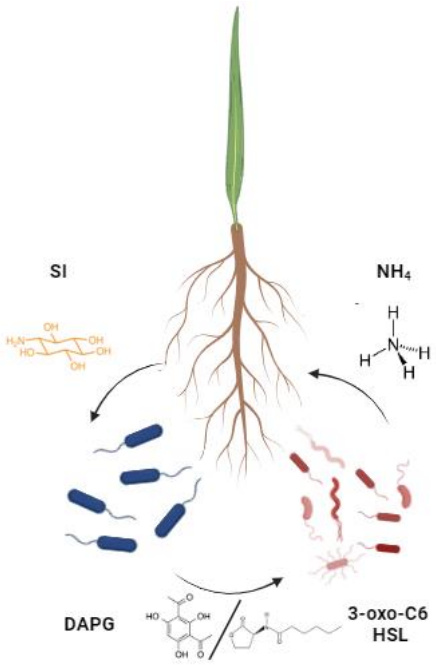


Figure 5.5 An illustration of bacterial relay signalling, using plant host derived rhizopine (SI) to induce compatible rhizobia (blue), which in-turn control a diverse bacterial community (red) through 2,4-DAPG/AHL signalling for PGP.

5.3 Engineering IAA Biosynthesis

5.3.1 Construction of IAA Biosynthesis Plasmid

To produce auxin in a heterologous bacterial host, the auxin pathway from *P. savastanoi* was cloned. *P. savastanoi* is a bacterial species known to secrete high concentrations of IAA during pathogenesis to induce hypertrophic growth on plant hosts to produce galls or knots on stems and leaves[172,173]. The indole acetamide pathway was used for auxin production as it is a well characterised pathway consisting of two enzymes, a tryptophan mono-oxygenase (IaaM) and indole-3-acetamide hydrolase (IaaH). Furthermore, other studies

have engineered this pathway to produce auxin synthesis in a variety of bacterial species, including rhizobia[299,300]. The genes for the indole acetamide pathway were domesticated, by the removal of internal type IIS restriction sites, by Dr Barney Geddes for utilisation with the Type IIS Golden Gate cloning pipeline for ease of use for design of synthetic[247,275]. The *iaaM* gene was amplified using primers oxp1339 and 1340, whilst *iaaH* was amplified using oxp1338 and oxp1341. The amplified genes were both stored in level 0 Golden Gate compatible plasmid pOGG072 to produce pL0M-SC-*iaaM* pOGG192 and pL0M-SC-*iaaH* pOGG193 respectively. Both IAA genes where then combined with characterised genetic parts to produce level 1 expression cassettes before combination of gene modules into a single level 2 plasmid. The *iaaM* gene was cloned into level 1 vector pOGG021 with a medium strength pL0M-P-*J23106* promoter (from plasmid pOGG121), RBS1 from plasmid pOGG144, and an F2S terminator from pOGG160. The *iaaH* gene was cloned into level 1 vector pOGG059 vector with a low strength *J23115* promoter from pOGG122, a RBSstd from pOGG143, and a DT15 terminator from pOGG156, producing two level 1 plasmids, the *iaaM* expression cassette in pOGG234, and the *iaaH* expression cassette in pOGG235. Different strength promotors were used to preserve the native expression ratio of IAA genes, as mRNA transcripts of *iaaM* to *iaaH* in *Pseudomonas* are approximately 10:1[173,247].

Initially, only constitutive IAA gene expression modules were constructed to allow the testing of IAA production in different bacterial strains, before going on to construct appropriate inducible cassettes compatible with the chosen strain's genetic background. Both level 1 plasmids pOGG234 and pOGG235 were combined into a single level 2 plasmid for divergent expression of *iaaM* and *iaaH*, one with the RK2 medium copy level 2 vector pOGG096 producing plasmid pOPS0667, and one in the high-copy number broad host range plasmid pOGG027 to produce pOPS0666 (*Figure 5.6*).

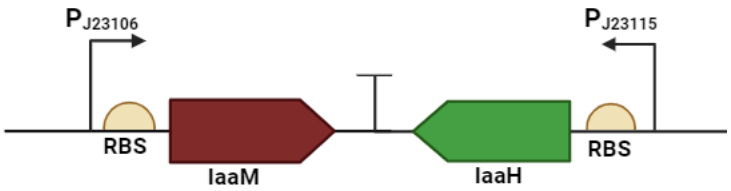


Figure 5.6 Diagram of auxin biosynthesis plasmids pOPS0666 and pOPS0667 encoding two genes tryptophan monooxygenase (IaaM) and indole-3-acetamide hydrolase (IaaH) from *P savastanoi*.

5.3.2 IAA Production Assay

To assay auxin production in bacterial strains, the constitutive IAA plasmids pOPS0667 and pOPS0666 were conjugated into the rhizobial strain *S. meliloti* CL150 producing OPS3103 and OPS3102 respectively. The production of IAA by CL150 strains was measured using the colorimetric Salkowski assay[298]. The Salkowski method has been widely used to detect IAA from microorganism cultures and is dependent on IAA complex formation with Fe^{3+} in the presence of a concentrated oxidizing acid. The coloured complex formed by this reaction shows a maximum absorbance at $\lambda = 530$nm (pink)[301,302]. The assay was adapted for high-throughput analysis by utilising a 96-well microplate format, whereby after 18 hours growth cultures were diluted to OD_{600} 1.0, 100 µL of bacteria culture supernatant was collected and combined with 200 µL of Salkowski reagent in triplicate. The plate was covered and incubated in the dark for 30 minutes, due to the light sensitivity of IAA, before measurement of absorbance at 530nm using a microplate reader. The absorbance value produced from incubation with just the culture media was subtracted from measurements to account for any background indole reaction. IAA production from *iaaH-iaaM* plasmids was compared to culture supernatants from the WT host strain as many rhizobia are known to produce varying levels of IAA[119]. As the amino acid tryptophan is the main precursor compound for IAA biosynthesis, production with our bacterial strains was tested with the addition of 1 mM

tryptophan to the culture media to test if this would produce a more visible phenotype through increased IAA production.

No significant difference was seen in the production of IAA measured by the colorimetric change between CL150 carrying pOPS0667 or pOPS0666 compared to the WT in the absence of tryptophan in the growth media. A difference was seen between strains with the inclusion of 1 mM tryptophan, as the pBBR1 plasmid pOPS0666 produced a significant increase in the production of IAA relative to WT CL150 ($p = 0.011$) (*Figure 5.7*), however, this increase was not biologically relevant as no meaningful increase in IAA production resulted from the strain carrying the plasmid, as the IAA background from WT CL150 was already so high. Addition of tryptophan to culture media resulted in higher IAA production with all three strains (*Figure 5.7*).

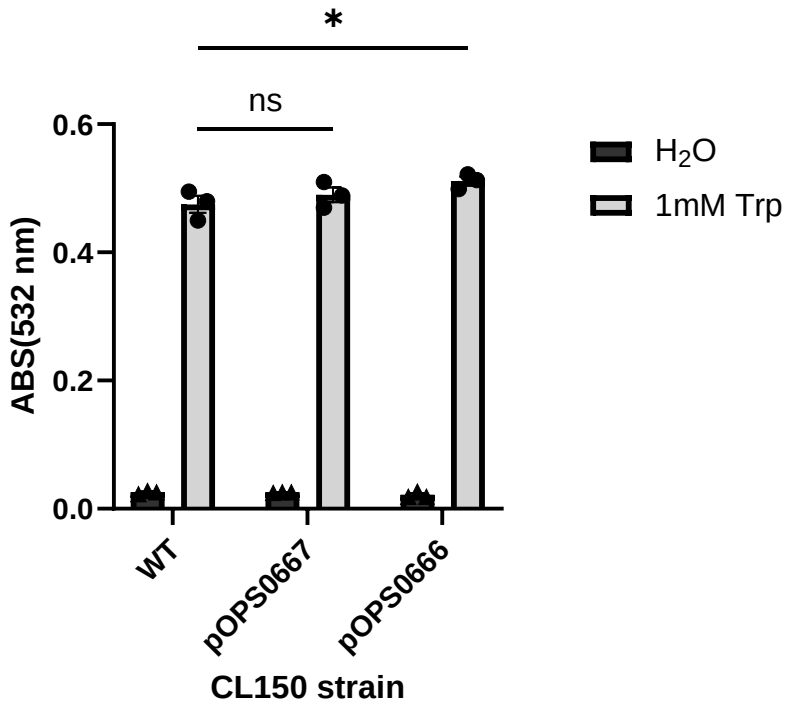


Figure 5.7 mean IAA production of CL150 strains carrying IAA plasmids pOPS0667 (RK2) and pOPS0666 (pBBR1) using 1:2 ratio of culture supernatant with Salkowski reagent and absorbance of the solution measured at 532 nm (± SEM, n = 3, two-way ANOVA with multiple comparisons, ns $p > 0.05$, * $p \leq 0.05$).

5.3.3 Rhizobial IAA Production

Clearly, as reported in the literature, rhizobia possess the ability to produce indole compounds either as a microbial signalling molecule or for microbe-plant interactions. Therefore, the ability of different WT strains held in the laboratory collection were assayed for their ability to produce IAA in-order to find a more suitable host to regulate IAA production. 15 bacterial species were assayed using the Salkowski assay discussed previously (*Figure 5.8*). Auxin is induced and synthesized through many conditions and different pathways between bacterial strains. At least six metabolic pathways for IAA biosynthesis have been proposed in bacteria, some of which may be tryptophan independent[303]. Therefore, the optimal environment and inducers required for productivity of auxin and its related metabolites varies.

This is reflected in the results of this assay, whereby a large variety in IAA production was seen across strains. With some such as AA4, A34 and *R. trifolii* producing large amounts of IAA (*Figure 5.8*). This relative increase could be due to higher affinity enzymes in biosynthetic pathways, or the positive feedback mechanisms regulated by IAA production such as the autoinduction of the *ipdC* gene transcription seen in *A. brasilense.* Furthermore, strains such as *B. japonicum* have been shown to not accumulate IAA in media due to the bacterium readily catabolising IAA which induces physiological changes affecting symbiosis[174].

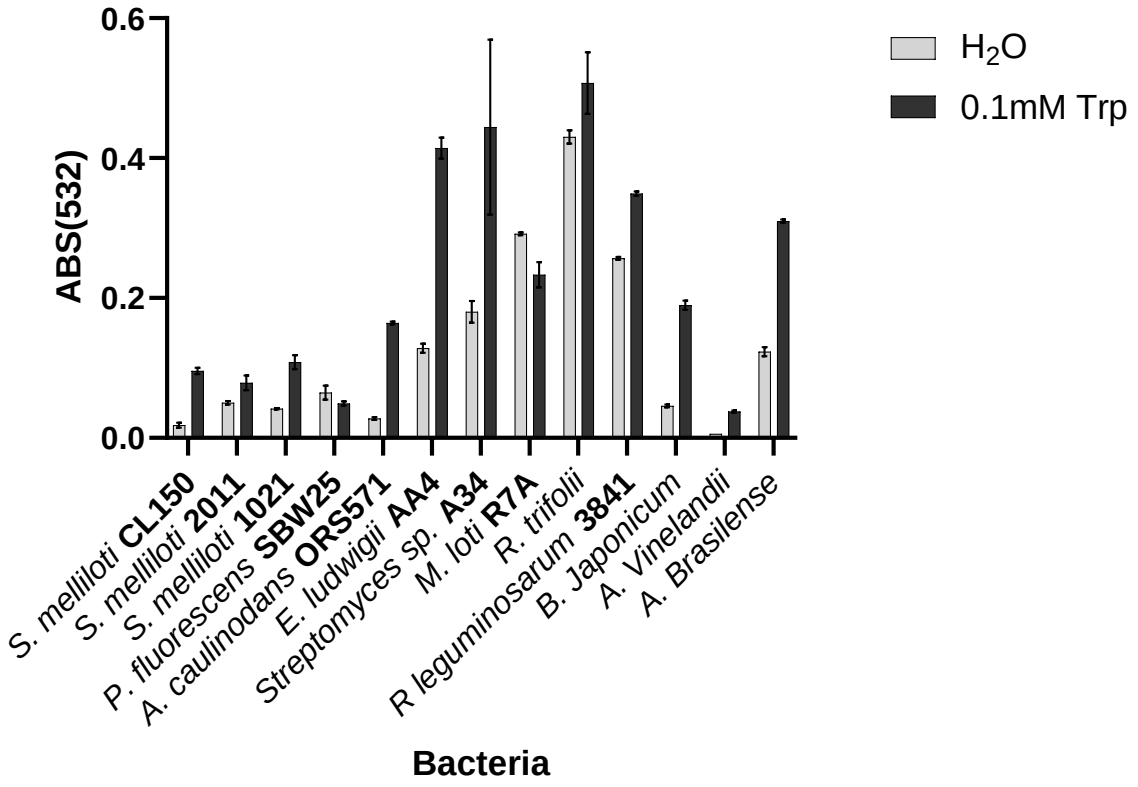


Figure 5.8 Mean IAA production of rhizobacterial species, incubated with and without the addition of 0.1 mM Tryptophan. Measured using 1:2 ratio of culture supernatant with Salkowski and absorbance of the solution measured at 532 nm (± SEM, n = 3).

5.3.4 *Pseudomonas* IAA Production

To test if the IAA genes clones in plasmids pOPS0666 and pOPS0667 would be more active in a more closely related bacterial strain, the plasmids were conjugated into *P. fluorescens* SBW25 (OPS3505 and OPS3504 respectively). SBW25 does not produce much IAA naturally (*Figure 5.8*) but is more closely related to *P. savastanoi* from which the *iaaM* and *iaaH* gene sequences were cloned. With *P. fluorescens* SBW25, there was no significant difference in the production of IAA from any strain with the addition of 0.1 mM tryptophan compared to H_2O. However, the RK2 plasmid pOPS0667 and the pBBR1 plasmid showed a significant increase in IAA production in both media relative to WT SBW25 ($p > 0.02$, and

p > 0.001 respectively) (*Figure 5.9*). As expected, the higher copy number pBBR1 plasmid pOPS0666 lead to the greatest production of IAA, demonstrating that IAA production can be engineered in rhizosphere bacteria with the addition of genes for the IAM biosynthetic pathway.

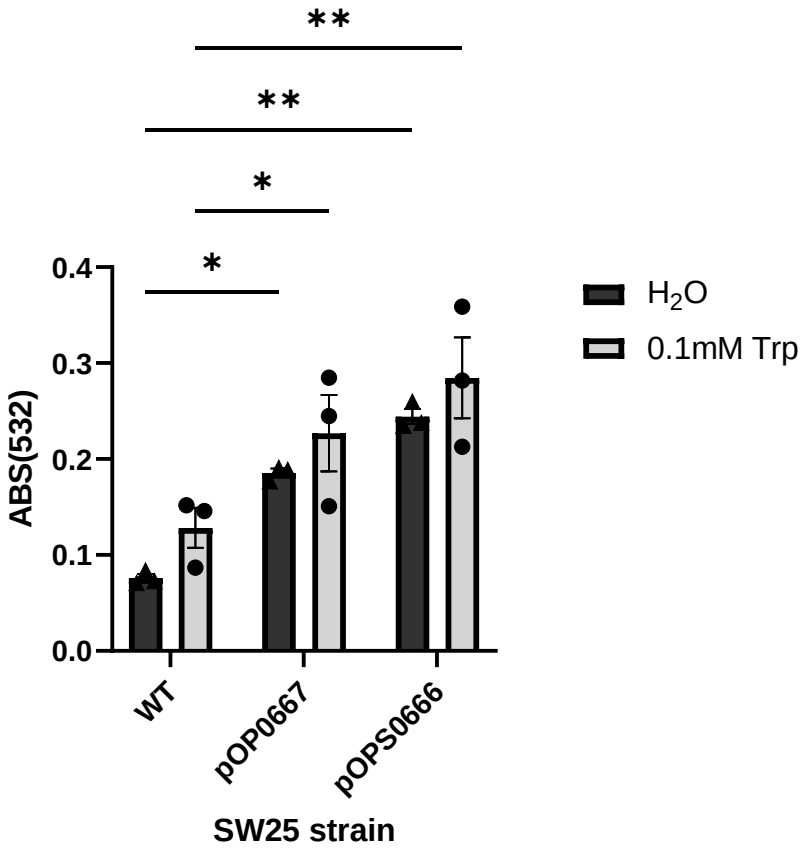


Figure 5.9 mean IAA production of SW25 strains carrying IAA plasmids pOPS0667 (RK2) and pOPS0666 (pBBR1) measured using 1:2 ratio of culture supernatant with Salkowski (± SEM, n = 3, two-way ANOVA with multiple comparisons, * $p \leq 0.05$, ** $p \leq 0.01$).

5.4 Engineering Bacterial Relay Signalling

5.4.1 2,4-DAPG Biosynthesis Constructs

To test if 2,4-DAPG could be used as a signal molecule from rhizobia to more diverse bacteria the *phl* genes responsible for biosynthesis in the bacterium *Pseudomonas fluorescens*

F113 were domesticated. *P. fluorescens* F113 is a PGP rhizobacterium isolated from the rhizosphere of sugar-beet and has been extensively studied for its secondary metabolite production as a candidate biocontrol agent against phytopathogens[297,304]. The *phl* genomic region of *P. fluorescens* consists of six open reading frames, one of which encodes *phlF* which acts as a repressor protein regulating the transcription of the *phl* operon by binding with an inverted repeated sequence *phlO* located downstream of the *phlA* transcriptional start site[305]. The *phlACBD* genes encode the acyltransferase at core of the operon, with *phlD* acting as a polyketide synthase responsible for production of monoacetylphloroglucinol (MAPG), a 2,4-DAPG precursor, which is converted to the final 2,4-DAPG product by *phlACB*. The operon also encodes a putative 2,4-DAPG permease gene, *phlE*[295,304,305]. The cloning strategy of Golden Gate cloning pipelines using type IIS enzymes was used to domesticate the *phl* operon by Dr Barney Geddes in this lab. The *phlACB* operon was domesticated using forward primers oxp1137-1140 and reverse primers oxp1141-1144 and the entire operon amplified using primers oxp2074 and oxp2075 for cloning into the pL0M plasmid pOGG072 to make the plasmid pL0M-*phlACB* (pOGG199). To ease assembly, the genes *phlD* and *phlE* were synthesized with internal type IIS restriction enzyme sites removed, the *phlD* gene was amplified with primers oxp2077 and oxp2078 and then cloned into the pOGG072 to make the plasmid pL0M-*phlD* (pOGG200). The *phlE* gene was similarly amplified with primers oxp1151 and oxp1152 and cloned into the level 0 plasmid pOGG072 to make plasmid pL0M-*phlE* (pOGG201).

A constitutive 2,4-DAPG biosynthesis plasmid was constructed using the domesticated *phl* genes by constructing three synthetic operons by level 1 Golden Gate cloning, before combing the *phlACB, phlD, and phlE* synthetic operons onto a single plasmid by level 2 cloning. Level 1 transcriptional units were constructed by expressing the domesticated *phl* genes from high strength *J23106* promoters with a standard RBS sequence and heterologous

terminators. The first synthetic operon combined the domesticated *phlACB* genes from pOGG199 were cloned into level 1 vector pOGG021 with P*J23106* from pOGG0121, RBSstd from pOGG143 and an F6 terminator to produce plasmid pOGG208. The second synthetic operon was constructed from the *phlD* open reading frame (pOGG200), a medium strength promoter P*J23106* (pOGG0121), pL0M-U-RBSstd (pOGG143) and pL0M-T-DT15S terminator (pOGG156) to produce plasmid pOGG215. The final level 1 plasmid combined the domesticated *phlE* gene (pOGG201) with the medium strength promoter P*J23106* (pOGG0121) with RBSstd (pOGG143) with pL0M-T-F2 terminator (pOGG160) to make plasmid pOGG214. The three level 1 plasmids pOGG208, pOGG214, and pOGG215 with expression cassettes *phlACB, phlD, and phlE* were combined in a level 2 Golden Gate reaction in the pL2V pOGG096 plasmid, carrying an RK2 origin of replication and kanamycin/neomycin selection gene and par operon, to make the full constitutive 2,4-DAPG biosynthesis plasmid pOPS0910 (*Figure 5.10*).

A rhizopine inducible 2,4-DAPG biosynthesis circuit was also constructed alongside constitutive plasmid pOPS0910. The same domesticated *phl* genes were organised into transcriptional units under control of SI inducible P*mocB,* the P*mocB* promoter was domesticated from the intergenic region between *mocR* and *mocB* from *S. meliloti* L530 into lv0 plasmid pOGG098. To produce synthetic transcriptional units, The *phlACB* genes from pOGG199 were cloned into level 1 vector pOGG024 with the SI inducible *PmocB* promoter from pOGG098, RBSstd from pOGG143 and an F6 terminator to produce plasmid pOGG208. The second transcriptional unit pOGG209 was produced from cloning P*mocB*, *phlD* from pOGG200, RBSstd from pOGG143, and an DT15 terminator from pOGG156 into PL1 vector pOGG054. Lastly, the *phlE* gene (pOGG201) with P*mocB,* RBSstd (pOGG143), and F2 terminator (pOGG160) were combined in PL1 vector pOGG035 to make plasmid pOGG213. As with pOPS0910, the three level 1 plasmids pOGG208, pOGG209, and pOGG213 with

PmocB inducible *phlACB, phlD, and phlE* expression cassettes were combined in a level 2 Golden Gate reaction in the pL2V pOGG096 plasmid, carrying an RK2 origin of replication and kanamycin/neomycin selection gene and par operon, to make the SI inducible 2,4-DAPG biosynthesis plasmid pOPS0909 (*Figure 5.10*).

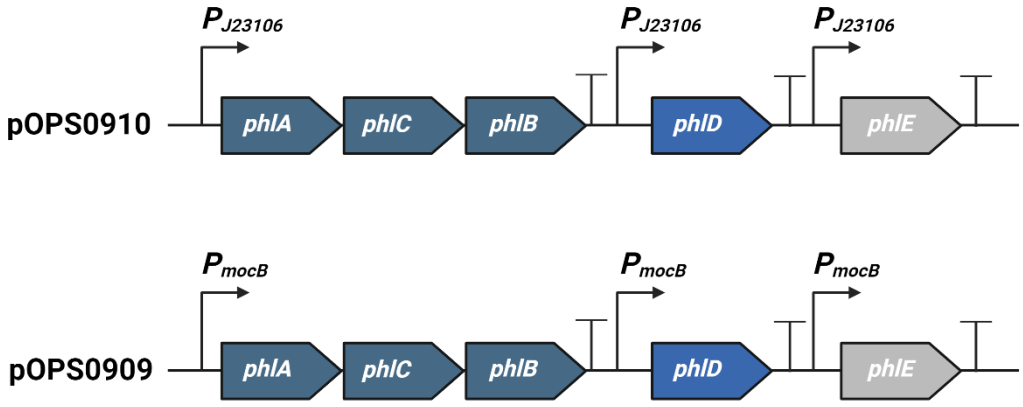


Figure 5.10 Constitutive 2,4-DAPG biosynthesis plasmid pOPS0910 (top) and rhizopine inducible 2,4-DAPG plasmid pOPS0909 (bottom) encoded on RK2 plasmids.

To analyse 2,4-DAPG production from biosynthetic strains, a 2,4-DAPG biosensor strain p148 was obtained by from Dr Minhyung Ryu of the Voigt Lab, MIT and stocked in our lab collection as pOPS1907[99]. The pOPS1907 biosensor is a pBBR1 based plasmid with a *sfGFP* reporter expressed from a P*phlA* promoter. The transcriptional regulator PhlF is expressed from a medium strength P*lacIq* constitutive promoter, thereby repressing *sfGFP* expression until the addition of 2,4-DAPG (*Figure 5.11*).

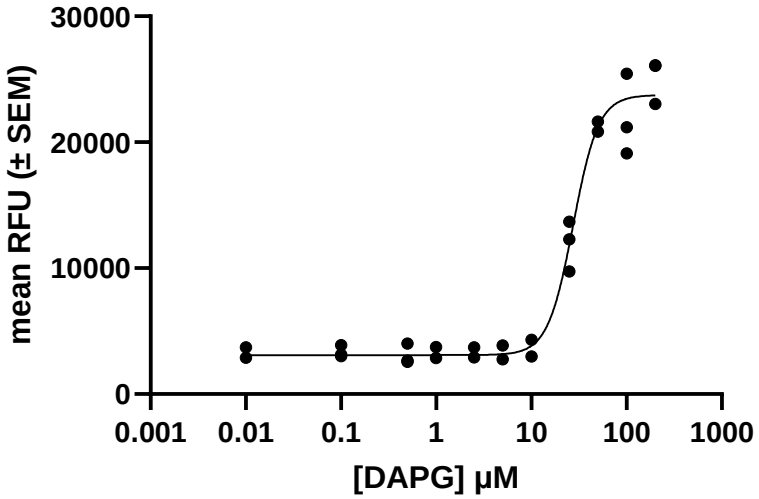


Figure 5.11 Standard curve of 2,4-DAPG inducible GFP biosensor pOPS1907 grown in triplicate in M9 media and induced with a range of 2,4-DAPG concentrations for 18 hours.

5.4.2 2,4-DAPG Production Assay

The rhizopine inducible 2,4-DAPG biosynthesis plasmid pOPS0909 was conjugated into *S. meliloti* strain OPS1864 carrying the *R. leguminosarum* 3841 landing pad with mini-Tn7 integrated rhizopine biosensor genes (P*J23115::mocBintBC-mocR*-P*mocB::gfp*), which is compatible with the RK2-PAR plasmid pOPS0909 (OPS3105). The integrated SI biosensor enables sensing of exogenous rhizopine and induction of *phl* genes from the P*mocB* promoters on pOPS0909. Production of 2,4-DAPG was compared to constitutive expression of *phl* genes from plasmid pOPS0910 which was conjugated into WT *S. meliloti* CL150 (OPS3104). The CL150 strains carrying the synthetic 2,4-DAPG biosynthesis plasmids were grown to an OD_{600} of 0.5 in 10 mL of TY media at 37˚C with 200 rpm shaking and the relevant antibiotics and induced with 10 µM SI for 18 hours. Cultures were then centrifuged at 4500g for 10 minutes at 4˚C to pellet the cells and debris and the supernatant collected. *E. coli* cells containing the 2,4-DAPG biosensor pOPS1907 were grown overnight in LB media supplemented with antibiotic. *E. coli* cultures were centrifuged at 4500g for 10 minutes at 4˚C to pellet the cells

and resuspended to OD_{600} 0.1 in M9 media. To measure induction, 400 µL of the resuspended cultures was added to a 96-deep well plate alongside 100 µL of CL150 strain culture supernatant. Combined cultures were incubated for 18 hours and then 100 µL transferred to a transparent 96-well plate for measurement of GFP fluorescence and OD_{600}. Subsequent fluorescence results were expressed as RFU and measured with three biological replicates for each CL150 2,4-DAPG biosynthesis strain. Induction from the *E. coli* biosensor was normalised by subtracting the background induction measured from the addition of supernatant from WT CL150.

Relay induced fluorescence results were compared to a 2,4-DAPG standard curve produced from the fluorescence of *E. coli* pOPS1907 incubated with a range of 2,4-DAPG concentrations. The constitutive 2,4-DAPG biosynthesis strain produced the greatest induction of the *E. coli* biosensor, with a mean RFU of 14882± 1531 which was three-fold greater than induction from the supernatant of strain carrying pOPS0909 induced with 10 µM SI, which gave a mean RFU of 3385± 992.7. The supernatant of strain carrying pOPS0909 when uninduced produced a mean induction of 1371± 144.0 in the *E. coli* biosensor, which is three-fold less than the RFU produced by SI induction of the strain ($p \leq 0.001$) (*Figure 5.12*). Comparison of induction by CL150 2,4-DAPG strains to the standard curve produced with the biosensor, normalised to induction with WT CL150 supernatant, gives an estimate of 340 µM of 2,4-DAPG produced by the constitutive biosynthesis plasmid pOPS0910 and < 20 µM by SI induction of pOPS0909. The supernatant of uninduced strains carrying pOPS0909 gives a response from the *E. coli* biosensor 10% above the background level, suggesting some leaky 2,4-DAPG production, however this value is outside the linear dynamic range of the standard curve and therefore it is difficult to estimate a concentration.

Despite the small increase in 2,4-DAPG production from pOPS0909 from addition of 10 µM SI, the inability to produce a higher concentration of 2,4-DAPG relative to the

constitutive plasmid pOPS0910 suggests that the single copy number of the *mocR* positive regulator or the rhizopine uptake by the cell were insufficient to induce transcription of *phl* genes under control of P*mocB* expression.

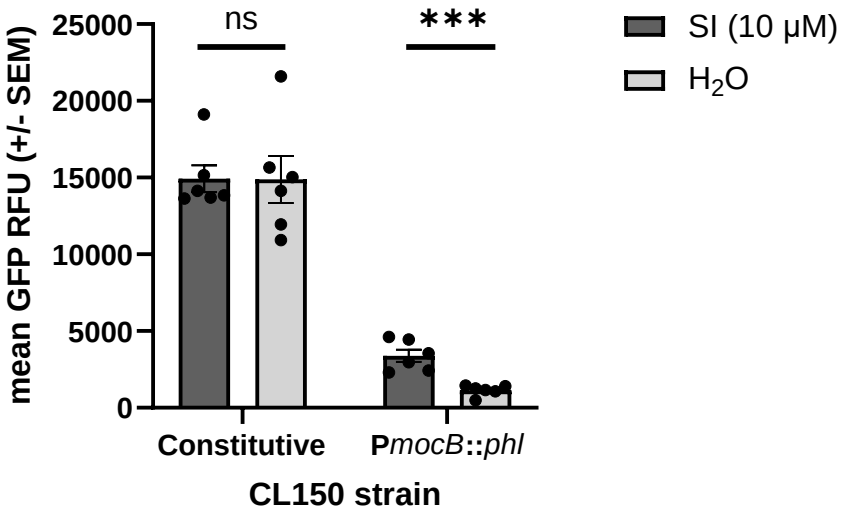


Figure 5.12 Induction of *E. coli* GFP biosensor incubated with a 1:5 dilution of supernatant from CL150 2,4-DAPG strains carrying the constitutive plasmid pOPS0910 and rhizopine inducible plasmid pOPS0909, with and without 10 µM SI. (± SEM, n = 6, results compared with independent Student's T-test, ns $p > 0.05$, *** $p \leq 0.001$).

The growth of *S. meliloti* 2,4-DAPG biosynthesis strains in UMS media containing 20 mM succinate and 10 mM ammonium chloride was measured to compare the effects of 2,4-DAPG biosynthesis on bacterial viability. Cultures of 2,4-DAPG biosynthesis strains were grown in UMS supplemented with 10 mM NH_4Cl and 20 mM succinate in triplicate in 24-well plates at 28°C with shaking over 50 hours. Rhizopine inducible 2,4-DAPG strain carrying pOPS0909 was supplemented with either 10 µM SI or H_2O. There was no significant difference between the mean generation time of strain carrying pOPS0909 in the presence or absence of 10 µM SI (4.767± 0.083 and 4.9± 0.246 respectively). However, CL150 strain carrying constitutive plasmid pOPS0910 showed a marked growth defect with a doubling time of 35.14±

2.49 hours suggesting constitutive expression of 2,4-DAPG imposed a significant metabolic burden upon the cell, or, that 2,4-DAPG production potentially reached toxic levels preventing growth (*Figure 5.13*).

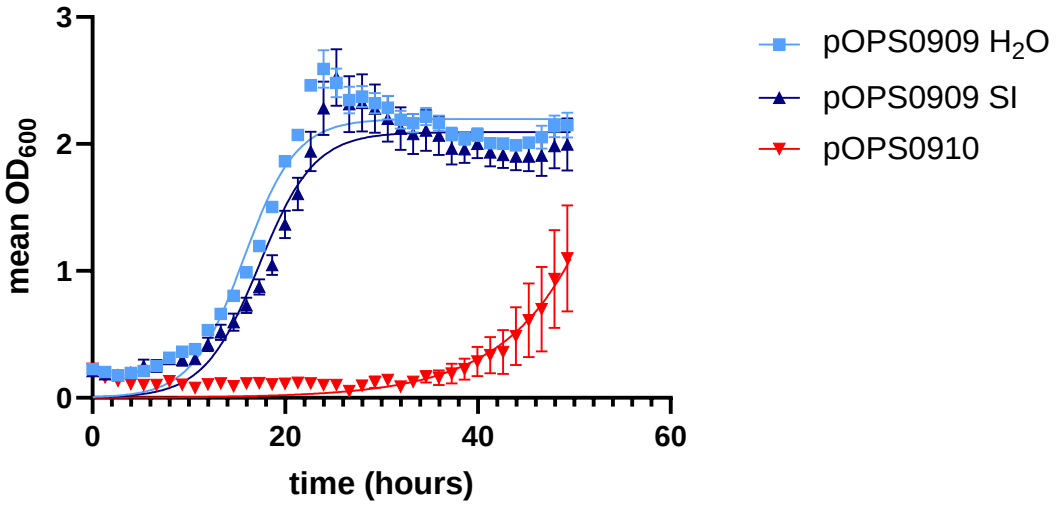


Figure 5.13 Mean growth curves of CL150 2,4-DAPG biosynthesis strains in UMS carrying the constitutive plasmid pOPS0910 (red) and rhizopine inducible plasmid pOPS0909, with and without 10 µM SI (dark and light blue respectively). (± SEM, n = 3).

5.4.3 Second Generation 2,4-DAPG Biosynthesis Plasmids

Data from Chapter 4 showed that induction of plasmid encoded P*mocB* in *Sinorhizobium* by chromosomally integrated SI transport components P*J23115::mocB-intBC* could be increased by adding a plasmid encoded *mocR* regulator, thereby increasing the copies of the transcription factor and expression in response to SI. To see if this strategy could also be used to increase induction of the 2,4-DAPG biosynthesis plasmid in response to SI, the transcriptional unit P*mocB::mocR* was amplified from rhizopine biosensor plasmid pOPS1052 using primer oxp5213/5214 and HiFi cloned into SmaI digested plasmid pOPS0909 to produce plasmid pOPS1847, which was confirmed by Sanger sequencing with oxp4238/4239. Plasmid pOPS1847 was then also conjugated into CL150 OPS1864 with integrated SI transporter genes.

Subsequent testing of 2,4-DAPG production by this strain in response to 10 µM SI showed no change in 2,4-DAPG production relative to the uninduced state as measured by the *E. coli* biosensor strain. Meanwhile, the previous 2,4-DAPG inducible plasmid pOPS0909 did show a significant, albeit small, increase in 2,4-DAPG production by SI ($p = 0.0066$) (*Figure 5.14*). Despite repeating the conjugation of pOPS1847 into OPS1864 multiple times and screening multiple colonies after each selection, no strain could be isolated which displayed induction of 2,4-DAPG in response to SI. This result suggests there was a problem with SI sensing by the strain, possibly due to silencing of the single copy SI transport genes[248]. Therefore, an alternative strategy to induce 2,4-DAPG production was pursued.

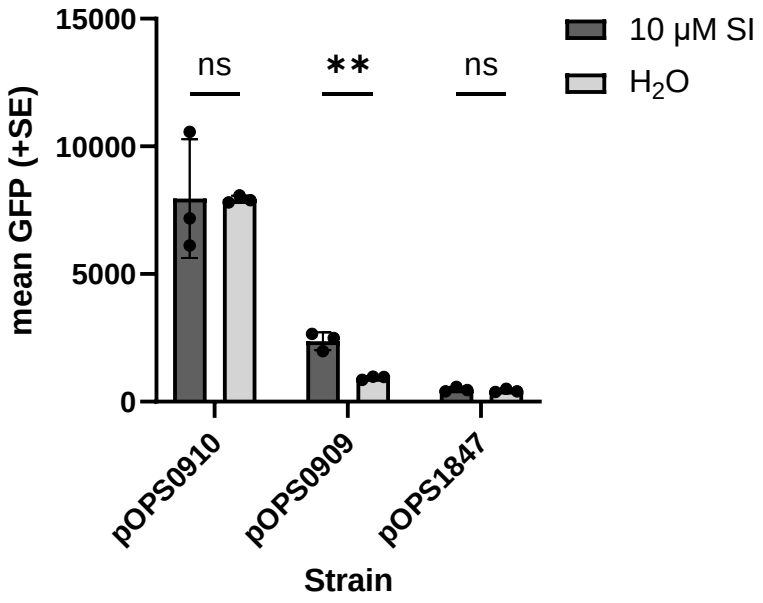


Figure 5.14 Induction of *E. coli* GFP biosensor incubated with a 1:5 dilution of supernatant from CL150 2,4-DAPG strains carrying the constitutive plasmid pOPS0910, SI inducible plasmid pOPS0909, and SI inducible plasmid pOPS1847 induced by integrated SI biosensor in OPS1864 with and without 10 µM SI. (± SEM, n = 3, independent Student's T-test, ns $p > 0.05$, ** $p \leq 0.01$).

To produce SI control of 2,4-DAPG production in CL150, the efficacious plasmids pOPS1052 and pOPS0889 were used as backbones to engineer P*mocB* control of *phl* gene expression. Primers oxp5635/2521 were used to amplify *phlACB*-P*mocB::phlD*-P*mocB::phlE* from 2,4-DAPG biosynthesis plasmid pOPS0909, and the fragment HiFi cloned into BamHI digested plasmid backbones pOPS1052 and pOPS0889. These constructs join the genes required for SI transport and induction alongside the *phl* biosynthesis genes under control of P*mocB* onto single plasmids with low and high copy numbers respectively. This cloning produced RK2 SI inducible 2,4-DAPG plasmid pOPS2093 and pBBR1 SI inducible 2,4-DAPG plasmid pOPS2095 (*Figure 5.15*), both plasmids were subsequently confirmed by sequencing with oxp2480/4046 and conjugated into WT CL150 to give strains OPS3511 and OPS3513 respectively. The induction assay of *E. coli* 2,4-DAPG biosensor pOPS1907 was then repeated with incubation of OPS3511 and OPS3513 for 24 hours and a 1:5 dilution of culture supernatant incubated with *E. coli* biosensor strain for 18 hours. pBBR1 plasmid pOPS2095 produced a two-fold greater induction of *E. coli* pOPS1907 compared to the RK2 biosynthesis plasmid pOPS2093 (mean RFU of 6574± 461 versus 3499± 228.6 respectively) (*Figure 5.15*). This induction equates to 200 μM and 150 μM of 2,4-DAPG produced by the respective cultures, reflecting the increase in copy number of *phl* genes in pBBR1 plasmid pOPS2095 relative to the low-copy RK2 plasmid pOPS2093. However, both biosynthesis plasmids displayed a marked increase in 2,4-DAPG production relative to expression of *phl* genes from pOPS0909 by an integrated SI biosensor (*Figure 5.15*).

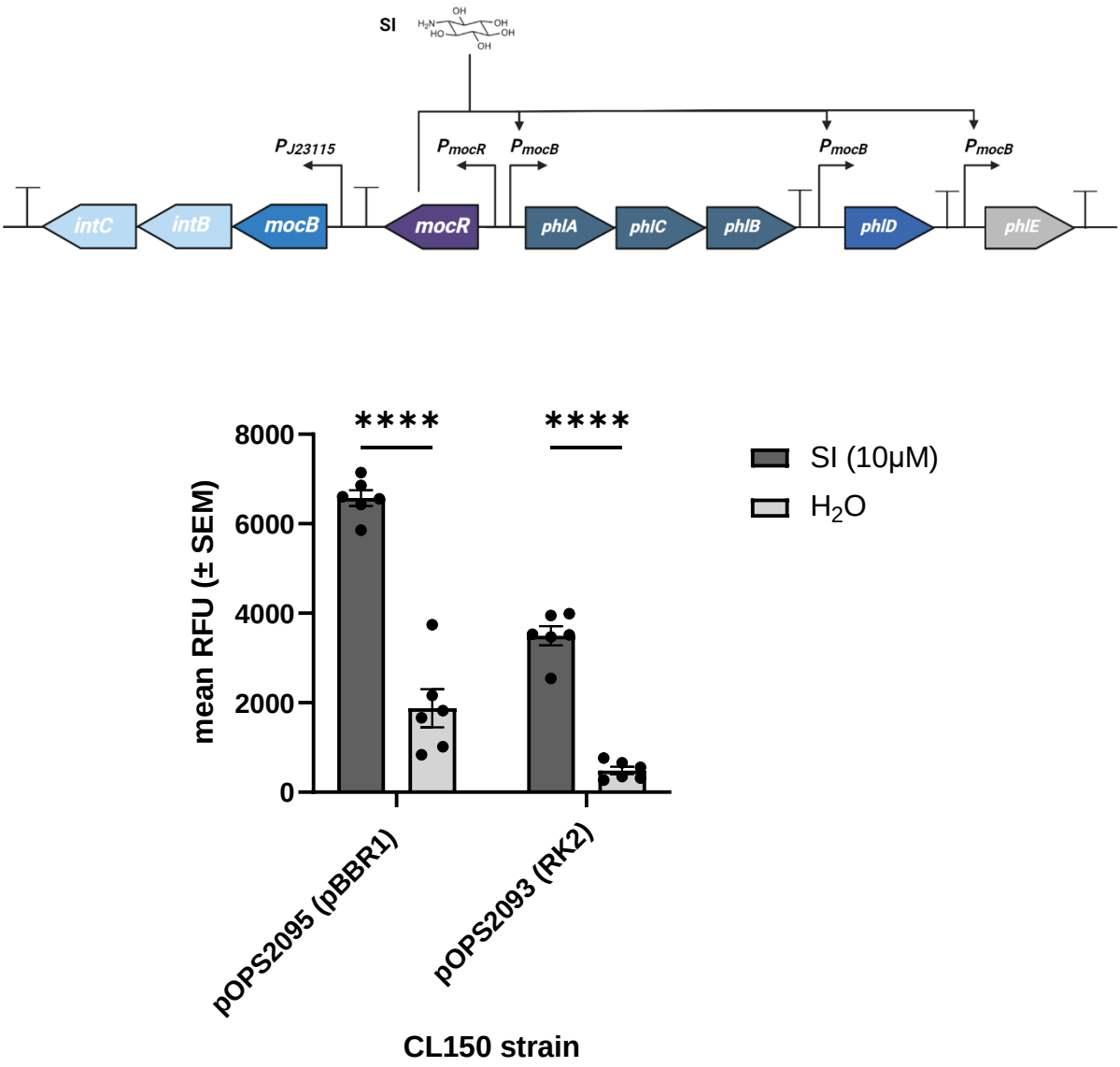


Figure 5.15 Top Diagram of SI induction of 2,4-DAPG biosynthesis in plasmids pOPS2095 and pOPS2093. **Bottom** mean induction of *E. coli* GFP biosensor incubated with a 1:5 dilution of supernatant from CL150 2,4-DAPG strains carrying SI inducible plasmids pOPS2095, and SI inducible plasmid pOPS2093 with and without induction by 10 µM SI. (± SEM, n = 6, independent Student's T-test, **** $p \leq 0.0001$).

Production of 2,4-DAPG by SI induction in CL150 was further increased by cloning the constitutive transcriptional unit for polyketide synthase *phlD*, required for the first step of phloroglucinol biosynthesis by condensation of three molecules of malonyl-CoA substrate. As well as the constitutive transcriptional unit for the putative permease *phlE,* which serves as an

export protein of toxic intermediates and therefore increases host resistance to 2,4-DAPG by preventing toxic build up or degradation intermediates in the host cell. The primers oxp2581/2582 were used to amplify *PJ23106::phlD- PJ23106::phlE* alongside *phlACB* from constitutive plasmid pOPS0910 and cloned into BamHI digested plasmid backbones pOPS1052 and pOPS0889 giving RK2 SI inducible 2,4-DAPG plasmid pOPS2094 and pBBR1 SI inducible 2,4-DAPG plasmid pOPS2096 (*Figure 5.16*), both plasmids were subsequently confirmed by sequencing with oxp2480/4046 and conjugated into WT CL150 to give strains OPS3512 and OPS3514 respectively. Supernatant from CL150 strain carrying pOPS2096 was grown in UMS media containing 10 µM SI was used to induce *E. coli* pOPS1907 and compared to the induction of pOPS2095 (pBBR1-P*mocB::phlACB*-P*mocB::phlD*-P*mocB::phlE*). The increased expression of *phlD* and *phlE* from constitutive promoter P*J23106* in pOPS2096 produced a 50% increase in induction of *E. coli* biosensor relative to pOPS2095 (mean RFU 9245± 520.1 and 6005± 197.2 respectively) (*Figure 5.16*). The increase in 2,4-DAPG production from CL150 carrying pOPS2096 resulted in a culture concentration of 240 µM. However, the increase in expression of *phlD* and the *phlE* permease also led to greater 'leaky' production of 2,4-DAPG by uninduced cultures (mean RFU 3169 ± 188.1), though addition of SI leads to a three-fold dynamic range in 2,4-DAPG production.

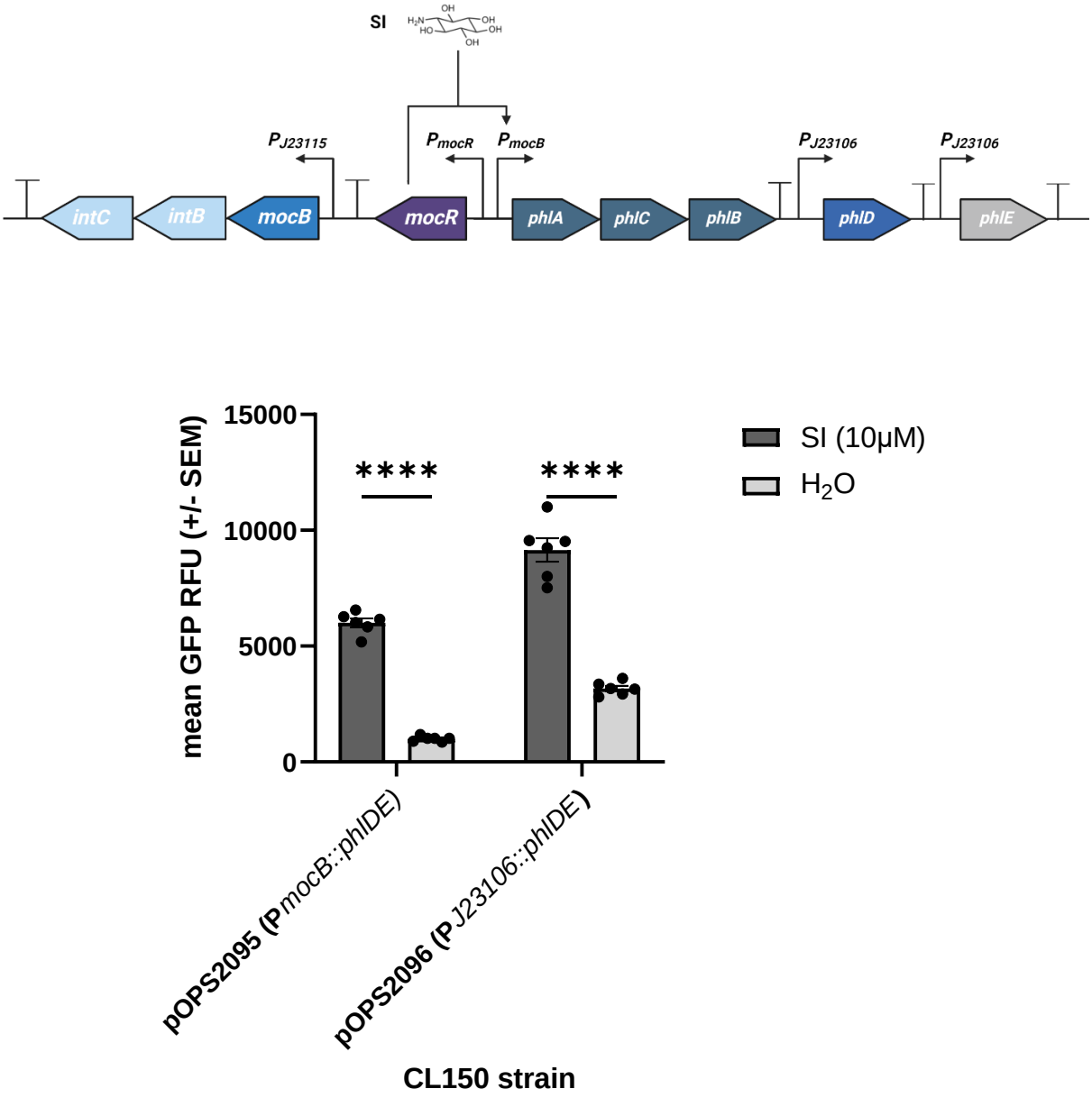


Figure 5.16 Top Diagram of SI induction of 2,4-DAPG biosynthesis plasmid pOPS2096 with constitutive production of *phlD* and *phlE*. **Bottom** mean induction of *E. coli* GFP biosensor incubated with a 1:5 dilution of supernatant from CL150 2,4-DAPG strains carrying SI inducible plasmids pOPS2095, and SI inducible plasmid pOPS2096, encoding constitutive *phlD* and *phlE* expression, with and without induction by 10 µM SI. (± SEM, n = 6, independent Student's T-test, **** $p \leq 0.0001$).

As measurements of 2,4-DAPG production by rhizopine from CL150 strains had been measured indirectly by collection of culture supernatants, an assay for direct cell-cell induction was needed to demonstrate the feasibility of relay signalling between bacterial consortia.

Firstly, a Lux biosensor was developed to measure 2,4-DAPG induction in non-alphaproteobacteria. This biosensor was required as the SI 2,4-DAPG circuits developed encode the *gfp* fluorophore, thereby necessitating an alternative biosensor to measure induction of relay strains in response to 2,4-DAPG production. Using domesticated synthetic parts from the laboratory collection a Lux reporter carrying *luxCDABE* genes domesticated from *Vibrio fischeri* was constructed for sensing 2,4-DAPG using Golden gate cloning[247]. The 2,4-DAPG inducible *phlF*-P*phlA* (pOGG137) was combined with *luxCDABE* operon (pOGG002) and DT16 terminator (pOGG157) in the pBBR1 vector pOGG024 to produce a luminescent biosensor pOPS1862, confirmed by sequencing using primers oxp0284/0283[306]. The Lux biosensor was carried in *E. coli* DH5α to test induction from 2,4-DAPG producing *S. meliloti* strains. Use of luciferase compared with fluorescent proteins has several benefits. Predominantly, bioluminescence background levels in living cells are extremely low, making bioluminescence up to 50 times more sensitive than fluorescence measurements[307].

E. coli carrying pOPS1862 was first tested for 2,4-DAPG induction using a standard curve of known 2,4-DAPG concentrations. *E. coli* was grown for 18 hours in LB broth with appropriate antibiotic before collection of cells by centrifugation and resuspension in LB to an OD of 0.1. *E. coli* was grown in 96 well plates in triplicate at 37°C for 18 hours with 2,4-DAPG concentration ranging from 0 to 200 µM. The *lux* biosensor pOPS1862 showed 1000-fold induction with 100 µM 2,4-DAPG over the uninduced state, producing a much greater dynamic range than the P*phlA::gfp* plasmid (*Figure 5.17*).

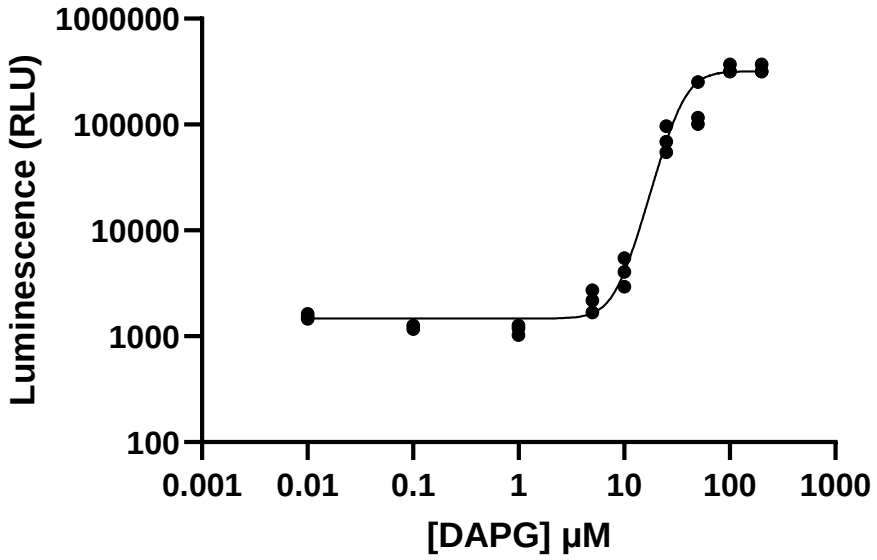


Figure 5.17 Lux luminescence of *E. coli* carrying pOPS1862 grown in triplicate in M9 media and induced with a range of 2,4-DAPG concentrations for 18 hours with luminosity (cps/OD_{600}) measured by GloMax luminometer (n = 3).

To measure direct induction of non-alphaproteobacteria by 2,4-DAPG production, an assay was developed using *E. coli* pOPS1862 as an indicator organism. Small 35 mm x 17.3 mm deep 16 mL microtiter plates were poured with 8 mL of TY with 1.5% agar, with or without 10 µM SI. Colonies of *S. meliloti* strains were streaked on TYA slopes with appropriate antibiotics and grown for 3 days at 28°C. After 5 days the culture was washed off the slope in 2 mL TY, centrifuged, and resuspended in 200 µL TY. The dense resuspension was used to spot 50 µL of culture (~ 10^9 cells) onto the centre of the 1.5% TYA plate surface and incubated for 48 hours. Subsequently, the microtiter plates and *Sinorhizobium* spot was covered with 6 mL of 1:1 mixture of stationary phase *E. coli* pOPS1862 and LB with 1.5% agarose. The *E. coli* and LB agarose mixture was kept in a water bath at 50°C, ensuring the agarose remained melted for pouring whilst allowing for survival of *E. coli* cultures. After incubating the plates overnight at 28°C, the luminescence of the *E. coli* bioreporter was measured using the GloMax luminometer with an integration period of 100 mS. Because induction was not uniform across

the LB agarose layer, the luminosity (cps) was divided by the area of the plate measured (mm) and normalised to luminosity when *E. coli* was incubated with WT CL150.

The pBBR1 2,4-DAPG biosynthesis pOPS2096 plasmid produced the greatest induction which was three-fold greater than the luminescence produced with the RK2 2,4-DAPG plasmid pOPS2095 (*Figure 5.18*). However, pBBR1 plasmid did produce an increase in *E. coli* luminescence in the absence of SI, relative to RK2, reflecting the leaky expression from P*mocB* promoters seen with *gfp* induction (*Figure 5.16*). Despite this, due to the large induction of recipient cells produced by SI induction of pOPS2096 in CL150, this strain will be used for experiments going forward.

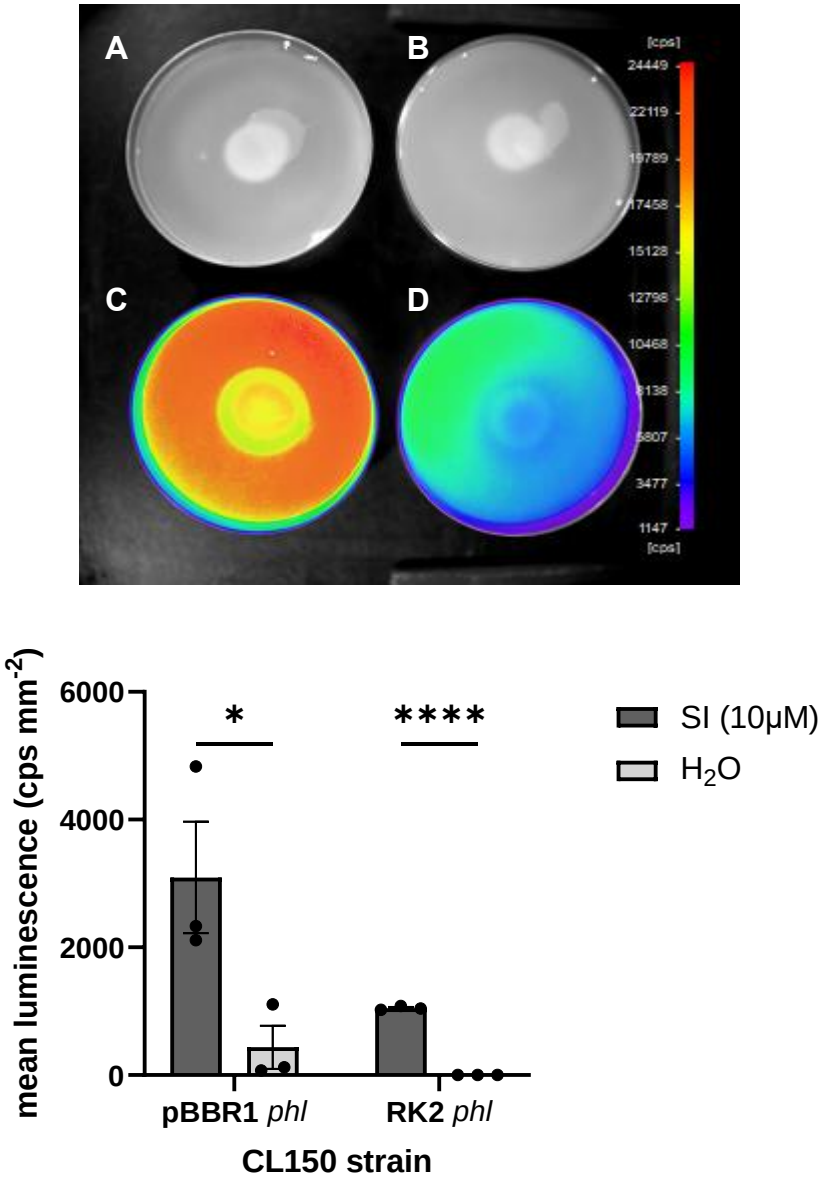


Figure 5.18 Top Example of *lux* induction of 2,4-DAPG inducible *E. coli* pOPS1862 in LBA above a rhizobial spot of CL150 WT on TYA with SI (A) and without SI (B), alongside 2,4-DAPG biosynthesis strain CL150 pOPS2096 with SI (C) and without SI (D). **Bottom** mean luminescence of *E. coli* pOPS1862 biosensor in LBA on top of concentrated spots of CL150 2,4-DAPG biosynthesis strains pOPS2096 (pBBR1-*PmocB::phlACB-PJ23106::phlD-PJ23106::phlE*) and pOPS2094 (RK2-*PmocB::phlACB-PJ23106::phlD-PJ23106::phlE*) on TYA with and without 10 µM SI. (± SEM, n = 3, independent Student's T-test, * $p \leq 0.05$, **** $p \leq 0.0001$).

5.4.4 Engineering AHL Relay Signalling

Alongside a rhizopine inducible 2,4-DAPG signal, an acyl-homoserine lactone (AHL) relay signal was developed. AHLs are well studied as quorum sensing signals and therefore make an excellent prospect for engineering a synthetic bacterial signal. To develop a rhizopine inducible AHL signal, the QS operon containing the LuxR-family transcriptional regulator *traR,* and AHL synthase *traI* from *Mesorhizobium* WSM1497 using primers oxp5633 and 5634. The corresponding fragment was then HiFi cloned into rhizopine biosensor plasmid pOPS1889, a pOPS1052 derivative plasmid with RK2 origin of replication but lacking *gfp* (*Figure 5.19*). The *traR* gene was amplified with its native RBS to minimise the autoinduction of P*traI* by the traR bound 3-oxo-C6-AHL. The corresponding SI inducible AHL plasmid pOPS2097, confirmed by sequencing with oxp2480/4046, was conjugated into WT CL150 to produce strain OPS3515.

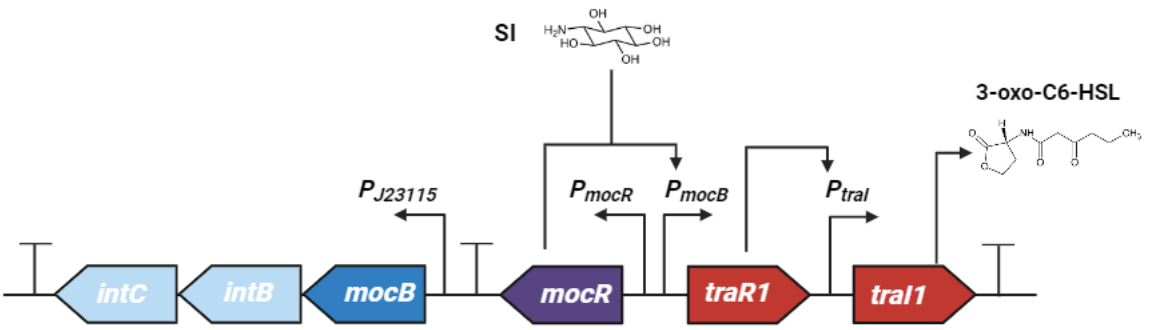


Figure 5.19 SI inducible plasmid pOPS2097 for 3-oxo-C6-HSL production carrying *traR* and *traI* genes amplified from *Mesorhizobium* WSM1497 under control of *PmocB*. Whereby, addition of rhizopine induces TraR expression which regulated expression of AHL synthetase TraI.

Sinorhizobium meliloti have been shown to produce AHLs required for the successful development of nodules and symbiosis, characterised by a *sinR/sinI* locus which produces AHLs with acyl chains ranging from 12 to 18 carbons[308]. To assay whether any native

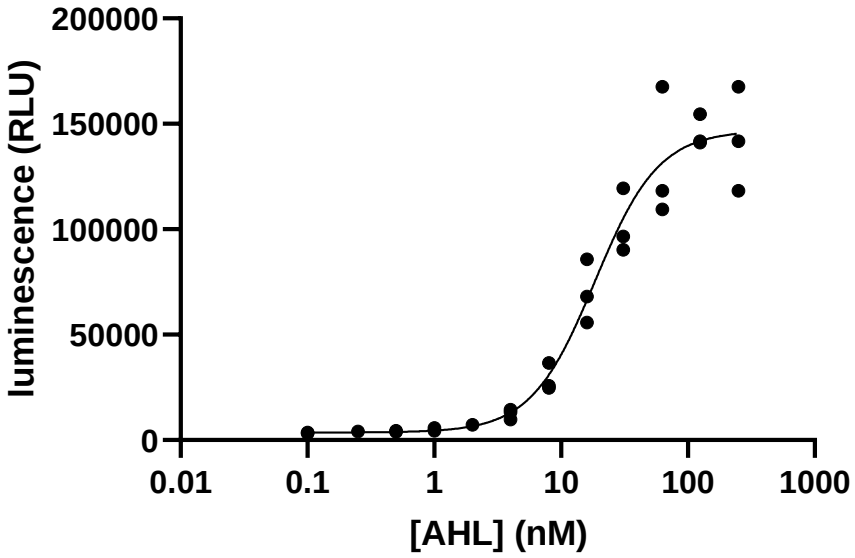
production of 3-oxo-C6-AHL occurred in CL150, the WT strain was streaked on non-selective TYA media adjacent to *Chromobacterium violaceum* CV026 which produces a purple pigment based on QS AHL signalling (*Figure 5.20*)[309]. CV026 violacein is inducible by AHLs which possess N-acyl side chains from C4 to C8 in length[309], no pigment was present upon co-inoculation of WT *Sinorhizobium* with CV026 on TYA.

Figure 5.20 WT CL150 (top) streaked adjacently to *Chromobacterium violaceum* CV026 (bottom) on UMA.

To measure SI induction of AHL in CL150, a Lux biosensor plasmid was produced using the luxR-P*lux* AHL inducible components from GFP plasmid pMR69 supplied by the Voight lab. The biosensor plasmid carried the LuxR transcriptional regulator, under control of the strong constitutive promoter P*lacIq*, which induces P*lux* expression. These AHL biosensor elements were amplified with oxp5713 and oxp5714 and cloned into NotI digested *luxCDABE* plasmid pOPS1917 to produce pOPS2158, confirmed by sequencing using primers oxp0284/0283 (*Figure 5.21*).

Utilising the AHL inducible *lux* plasmid in *E. coli*, an assay could be performed based off the method developed for detection of 2,4-DAPG secretion of CL150. Whereby, direct

induction of non-alphaproteobacteria by an AHL signal could be measured in a high-throughput manner with biological replicates. Therefore, luminescence 96-well plates were poured with 180 µL of TY with 1.5% agar, with or without 10 µM SI. Colonies of *S. meliloti* strains were streaked on TYA slopes with appropriate antibiotics and grown for 3 days at 28°C. After 3 days the culture was washed off the slope in 2 mL PBS, centrifuged, and resuspended in 200 µL TY. The dense resuspension was used to spot 50 µL of culture (~ 10^9 cells) onto the centre of the 1.5% TYA plate surface and incubated for 48 hours. Subsequently, the microtiter plates and *Sinorhizobium* spot was covered with 130 µL of 1:1 mixture of stationary phase *E. coli lux* biosensor pOPS2158 and LB with 1.5% agarose. The *E. coli* and LB agarose mixture was kept in a water bath at 50°C, ensuring the agarose remained melted for pouring whilst allowing for survival of *E. coli* cultures. After incubating the plates overnight at 28°C, the luminescence of the *E. coli* bioreporter was measured using the GloMax luminometer with an integration period of 100 mS (*Figure 5.21*). The luminosity (cps) was divided by the area of the well (mm) and normalised to luminosity when *E. coli* was incubated with WT CL150.



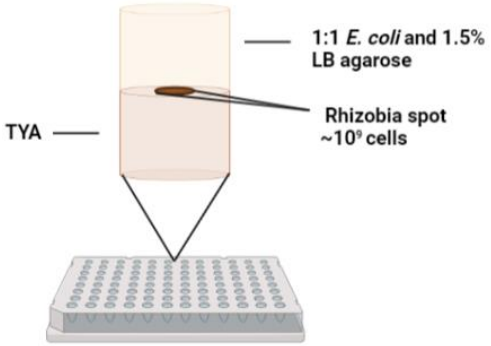


Figure 5.21 Top Induction of *E. coli* pOPS2158 *luxCDABE* biosensor induced with 3-oxo-C6-HSL incubated in triplicate for 18 hours in LB, with luminosity (cps/OD_{600}) measured by GloMax luminometer (n = 3). **Bottom** Assay setup to measure direct induction of *E. coli* pOPS2158 by CL150 AHL relay strains. CL150 were grown in TYA and concentrated 100-fold in 50 ul UMS and spotted on top of TYA. An *E. coli* pOPS2158 culture mixed 1:1 with 1.5% agarose was poured over and left to incubate for 18 hours. The resulting luminescence was measured by GloMax luminometer.

Using this assay, no difference was observed in *E. coli* luminosity with CL150 SI inducible AHL strains in the presence or absence of SI (*Figure 5.22*). With the *S. meliloti* strain producing a constitutive AHL phenotype. The constitutive activity indicates that the leaky expression observed from P*mocB* in SI plasmids lead to expression of *traR* and subsequently

traI, which synthesises the autoinducer 3-oxo-C6-AHL. The autoinducer then binds to TraR, further activating *traI* expression and AHL synthesis.

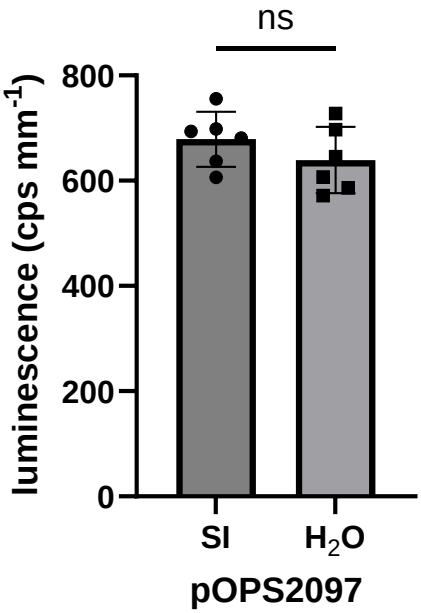


Figure 5.22 Induction of *E. coli* pOPS2158 *luxCDABE* biosensor by AHL 3-oxo-C6-HSL produced by CL150 carrying pOPS2097 incubated together in a 96 well plate, with luminosity (cps mm^{-1}) measured after 18 hours incubation (n = 6, ±SEM, independent Student's T-Test, ns $p > 0.05$).

To mitigate the autoinduction of the *traR-traI* biosynthesis genes, SI plasmids were constructed encoding a weak RBS previously characterised in this lab. Primers oxp5757 and oxp5634 were used to amplify *traR-traI,* with the native RBS of *traR* replaced by weak RBS28. The subsequent biosynthesis operon was again HiFi cloned into pOPS1889 plasmid to give plasmid pOPS2099, which was confirmed by sequencing with oxp2480/4046. This plasmid was transferred into WT CL150 by conjugation to give strain OPS3517 (*Figure 5.23*). The *E. coli lux* induction overlay experiment was repeated with this new AHL biosynthesis *S. meliloti* strain. The reduced translation of TraR did produce a significant reduction in AHL induced *lux* expression of *E coli* in the absence of rhizopine (*Figure 5.23*). However, this strain still

produced high levels of AHL in the absence of the SI inducer when grown in the presence of the *E. coli lux* biosensor.

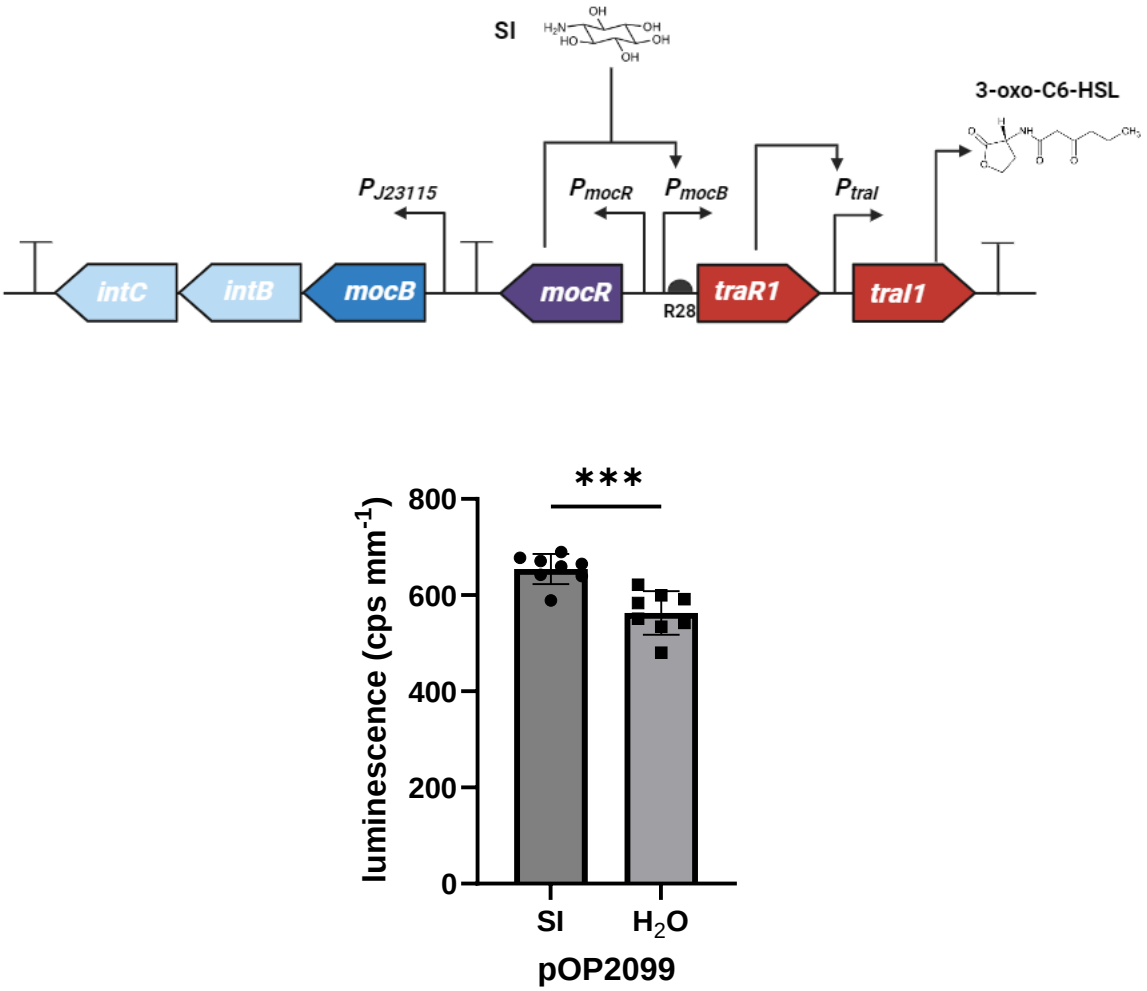


Figure 5.23 Top Diagram of SI inducible AHL biosynthesis plasmid pOPS2099 with the weak RBS R28 placed in front of TraR. **Bottom** Induction of *E. coli* pOPS2158 *luxCDABE* biosensor by AHL 3-oxo-C6-HSL produced by CL150 carrying pOPS2099 incubated together in a 96 well plate, with luminosity (cps mm^{-1}) measured after 18 hours incubation (n = 8, ±SEM, independent Student's T-Test *** $p \leq 0.001$).

It is therefore indicative that the positive-regulation of TraR by the 3-oxo-C6-HSL product, though it leads to a large production of AHL, minimises the dynamic range of the relay signal due to marked leaky expression. Therefore, a third SI inducible AHL biosynthesis plasmid was produced. To remove the autoinduction response, only the AHL synthase gene

traI was placed under direct control of the P*mocB* promoter, with the gene and its native RBS amplified by primers oxp5898 and oxp5899 and HiFi cloned into BamHI digested pOPS1889 to give plasmid pOPS2100, which was confirmed by sequencing with oxp2480/4046 (*Figure 5.24*). The P*mocB::traI* plasmid was conjugated into *S. meliloti* CL150 to produce OPS3518. This strain only produced 60% induction of the *E. coli lux* biosensor strain relative to the inducible *traR-traI* biosynthesis strain, equivalent to approximately 75nM 3-oxo-C6-HSL produced. The observed reduction in AHL biosynthesis is likely due to removal of the autoinduction promotion of expression from P*traI* by TraR, but this removal produced a 7.5-fold reduction in *E. coli lux* expression in the absence of SI (*Figure 5.24*). Therefore, the P*mocB::traI* plasmid demonstrates a more binary relay signal for control of recipient bacterial gene expression.

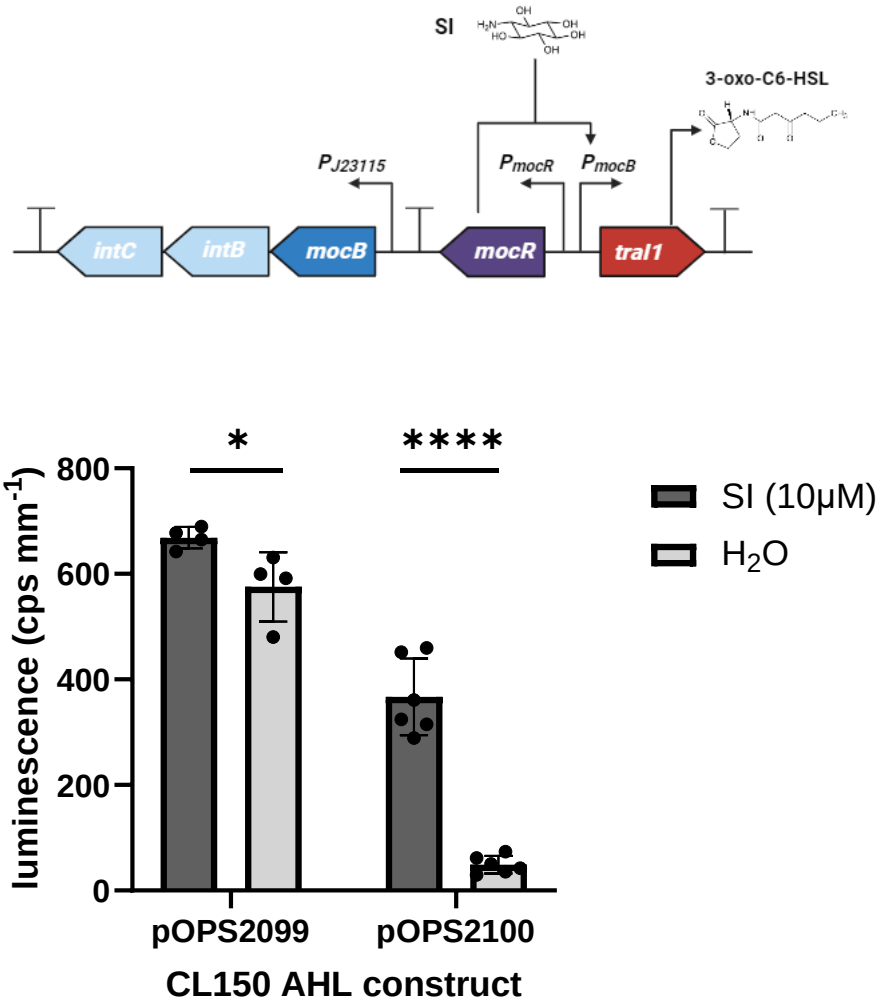


Figure 5.24 Top Diagram of SI inducible AHL biosynthesis plasmid pOPS2100 with P*mocB* induction of AHL synthase *traI*. **Bottom** mean *lux* Induction of *E. coli* pOPS2158 biosensor by AHL 3-oxo-C6-HSL produced by CL150 strains carrying pOPS2099 and pOPS2100 respectively, incubated with *E. coli* in a 96 well plate with luminosity (cps mm^{-1}) measured after 18 hours incubation (n = 6, ±SEM, multiple independent Student's T-Test, * $p \leq 0.05$ **** $p \leq 0.001$).

5.4.5 Relay Signalling to PGP Bacterial Species

To test the ability of both 2,4-DAPG and AHL relay signal strains to induced expression in non-alphaproteobacteria, relay induction from CL150 2,4-DAPG producing strain OPS3514 and AHL producing strain OPS3518 was used to induce cultures of *E. radicincitans.* Alongside this biologically relevant inoculant for associative nitrogen fixation in cereals, the alpha-

proteobacteria *A. caulinodans,* which has previously been demonstrated to be a good candidate for PGP[126], was also used to test SI inducible relay induction from CL150. A plasmid for 2,4-DAPG inducible expression of *gfp* has already been developed in this Laboratory, pOPS1537. An AHL inducible *gfp* biosensor was made to measure expression of the AHL relay signal in *E. radicincitants* and *A. caulinodans.* To compare expression between the two signals an RK2 backbone, pOGG093, was digested with BamHI, and primers oxp6029/6030 and oxp5882/5883 were used to amplify *luxR-Plux* and *gfp* respectively to produce AHL biosensor plasmid pOPS2155 via HiFi cloning, the resulting plasmid was confirmed by sequencing with oxp0283/284 (*Figure 5.25*). This plasmid was conjugated into *E. radicincitans* (OPS3689) and *A. caulinodans* ORS571 (OPS3691).

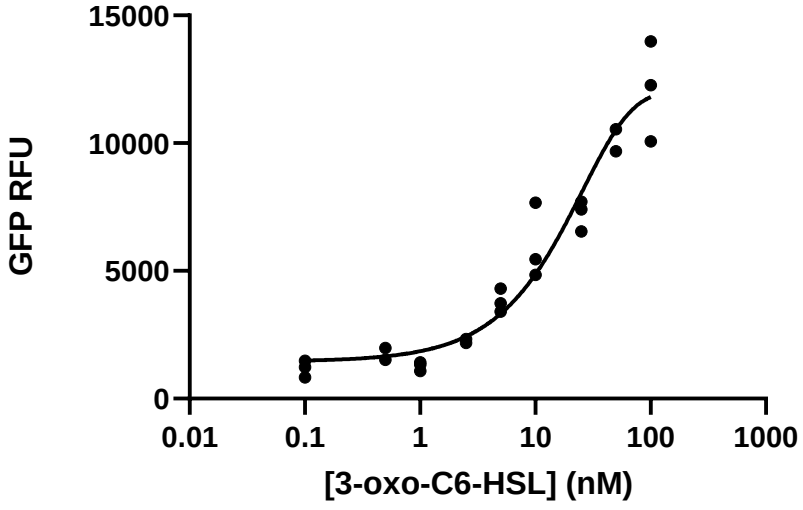


Figure 5.25 GFP fluorescence of *E. coli* pOPS2155 biosensor induced by AHL 3-oxo-C6-HSL, *E. coli* were induced at OD_{600} 0.3 and incubated for 18 hours in M9 media with AHL before measurement of fluorescence (n = 3).

To measure induction of gene expression by AHL and 2,4-DAPG relay signals in associative nitrogen fixing strains, *E. radicincitans* and *A. caulinodans* carrying the 2,4-DAPG and AHL inducible *gfp* plasmids (pOPS1537 and pOPS2155 respectively) were grown

overnight on UMS, alongside *S. meliloti* P*mocB::traI* (pOPS2100) and P*mocB::phlACB*-P*J23106::phlDE* (pOPS2096) with 10 μM SI. The supernatant from induced CL150 cultures was collected and used to induce respective reporter strains diluted to OD_{600} 0.5 in a 1:5 ratio. Mixed cultures were incubated for 18 hours before measuring the subsequent fluorescence by microplate assay (*Figure 5.26*).

Induction of *gfp* by 3-oxo-C6-HSL in *E. radicincitans* showed a 6.8-fold increase versus a 12.7-fold increase in *A. caulinodans*. Similarly, both strains showed five-fold and 8.5-fold induction respectively in the presence of 2,4-DAPG produced by *S. meliloti*. This result demonstrates non-alphaproteobacteria are capable of responding to both relay signals developed, unlike rhizopine (*Figure 5.26*).

The next step of demonstrating the feasibility of a relay signal to control PGP traits is to induce a measurable phenotype which could potentially provide a benefit to the cereal species. The obvious PGP-trait to control in this case is associative nitrogen fixation. We have previously demonstrated the ability to control N_2 fixation in *A. caulinodans,* however, this has so far not been demonstrated in *E. radicincitans,* due to its inability to respond to rhizopine.

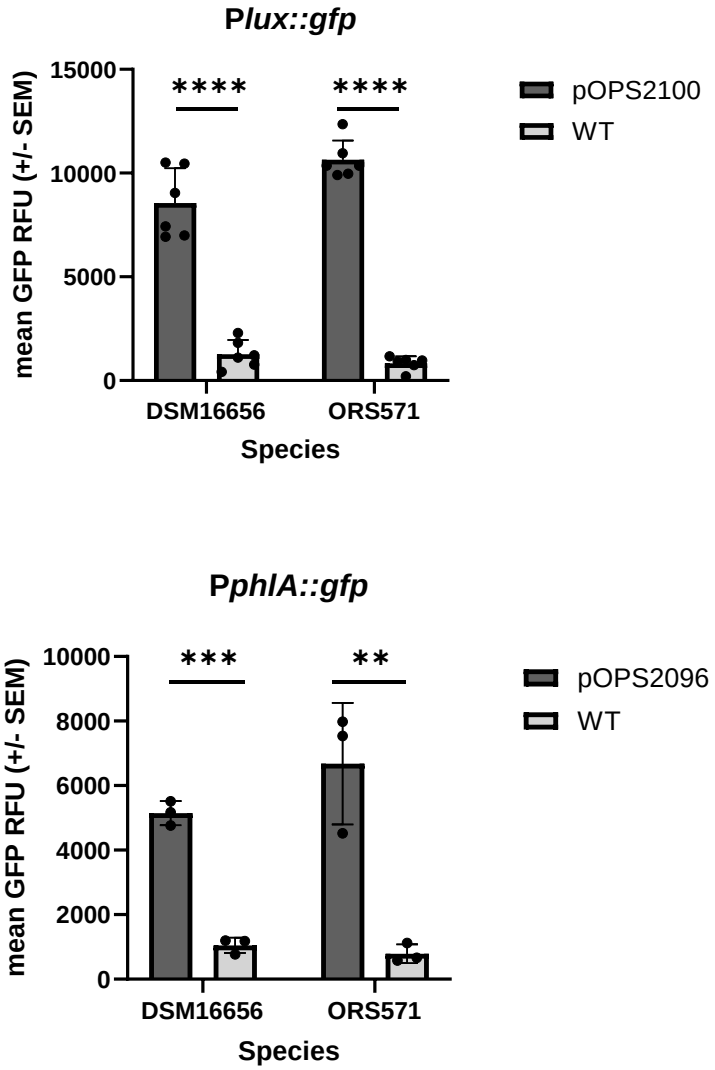


Figure 5.26 Top Induction of *gfp* in *E. radicincitans* DSM16656 and *A. caulinodans* ORS571 carrying AHL biosensor pOPS2155 (P*lux::gfp*) incubated 1:5 with supernatant collected from CL150 WT or CL150 with AHL biosynthesis plasmid pOPS2100 induced with 10 µM SI. Cultures were incubated for 18 hours and the subsequent *gfp* expression measure by microplate (n = 6, ±SEM, multiple independent Student's T-Test, **** $p \leq 0.0001$). **Bottom** Induction of *gfp* in *E. radicincitans* DSM16656 and *A. caulinodans* ORS571 carrying 2,4-DAPG biosensor pOPS1537 incubated 1:5 with supernatant collected from CL150 WT or CL150 with 2,4-DAPG biosynthesis plasmid pOPS2096 (P*phlA::gfp*) induced with 10 µM SI. Cultures were incubated for 18 hours and the subsequent *gfp* expression measure by microplate (n = 3, ±SEM, multiple independent Student's T-Test, ** $p \leq 0.01$, *** $p \leq 0.001$).

5.4.6 *E. radicincitans nifLA* Deletion

Whilst an *A. caulinodans* Δ*nifA* deletion mutant has already been developed in this laboratory, we aimed to replicate this in *E. radicincitans* to demonstrate control of N_2 fixation. It has been previously demonstrated that a deletion of *nifL* in *Azotobacter vinelandii* leads to a constitutive N_2 fixation phenotype and ammonium secretion[215]. In *E. radicincitans* the *nifLA* operon directly regulates the rest of the *nif* cluster through transcriptional activation by NifA and nitrogen and oxygen dependent repression of NifA by NifL. Therefore, alongside Bergthor Traustason, three mutants were produced in *E. radicincitans, ΔnifL* (OPS3472)*, ΔnifA* (OPS3473)*, and ΔnifLA* (OPS3474). The PK19mobSacB vector pOPS1487 was used to produce mutants in this strain. After mobilization into the recipient strain, the PK19mobSacB plasmid can only be maintained by integration into the host chromosome via homologous recombination, based on 1Kb sequences taken from upstream and downstream of the desired gene to be knocked out, cloned into the plasmid digested by BamHI. Excision of the intervening plasmid sequence by a double cross-over event can be selected for on 10% sucrose. For deletion of *nifL* in *E. radicincitans* primers oxp5910 and 5911 were used to amplify the upstream region whilst oxp5912 and oxp5913 amplified the downstream region, leaving *nifA* in-frame with the native promoter. For Δ*nifA* primers oxp5914, 5915, 5916, and 5917 were used, whilst deletion of the whole *nifLA* operon used primers oxp5918, 5919, 5920, and 5921. Deletion of the relevant *nif* regulatory genes were confirmed by PCR followed by sequencing using primers oxp5922-5923 which bind the genomic region outside the *nifLA* operon.

To screen if deletion of *nifL* or *nifA* resulted in an altered nitrogen fixation phenotype in *E. radicincitans,* mutants were grown in LB overnight before centrifuging the cells and resuspending to OD_{600} 0.5 in UMS with 10 mM glucose and vitamin solution. The resulting cultures were incubated for 3 hours at 3% O_2 before sealing the cultures and performing an acetylene induction assay over 6 hours. OPS3472, the Δ*nifL E. radicincitans* strain, was still

able to fix nitrogen albeit at 73% the rate of the WT strain. No nitrogen fixation was observed in Δ*nifA* or Δ*nifLA* strains showing that deletion of *nifA* abolishes expression of *nif* genes as expected (*Figure 5.27*).

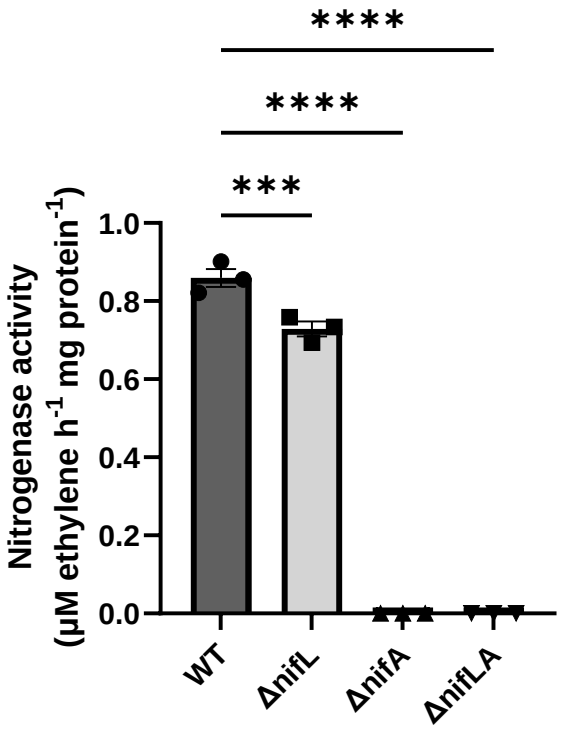


Figure 5.27 ARA of *E. radicincitans* mutants *ΔnifL* (OPS3472)*, ΔnifA* (OPS3473)*, and ΔnifLA* (OPS3474) relative to WT*,* performed in UMS with 10 mM glucose and vitamin solution. The resulting cultures were incubated for 3 hours at 3% O_2 before sealing the cultures and performing an acetylene reduction assay over 6 hours. (n = 3, ±SEM, one-way ANOVA with Tukey's multiple comparisons, *** $p \leq 0.001$, **** $p \leq 0.0001$).

5.4.7 Relay Inducible NifA Controllers

To control N_2 fixation in *E. radicincitans* via *S. meliloti* relay signals, two DSM16656 *nifA* inducible plasmids were constructed. To control *nifA* via 2,4-DAPG, the *phlF*-P*Phl*::*gfp* plasmid pOPS1537 was digested with XbaI, and the *nifA* gene from *E. radicincitans* amplified alongside the native RBS using primers oxp6049 and 6050 producing 2,4-DAPG inducible

nifA plasmid pOPS2154. Similarly, to construct an AHL inducible *nifA* plasmid, *luxR*-P*lux::gfp* plasmid pOPS2155 was also digested with XbaI and the RBS-*nifA* from DSM16656 amplified by oxp6053-6054 for construction of the controller via HiFi cloning, producing plasmid pOPS2153. Both plasmids were confirmed by sequencing with oxp283/284 and conjugated into DSM16656 Δ*nifLA* strain OPS3474 to produce AHL inducible OPS3681 and 2,4-DAPG inducible OPS3682 *nifA* strains.

5.4.8 *In Vitro* Relay Induction of Nitrogen Fixation

Both plasmids were tested for the ability to control N_2 fixation *in vitro* via ARA at 3% oxygen. *E. radicincitans* strains OPS3681, OPS3682, alongside WT DSM16656 were grown overnight in LB before centrifugation to pellet the cells and washing thrice in UMS. Cells were then resuspended to a final volume of 5 mL at an OD of 0.5 before addition of 100 nM 3-oxo-C6-HSL to strain OPS3681 and 20 µM 2,4-DAPG to OPS3682 to induce nitrogen fixation. Cultures were then incubated for 6 hours in an anaerobic chamber before measuring the relative ethylene/acetylene ratio. AHL inducible *nifA* cultures of DSM16656 produced N_2 fixation values which were 98% of WT fixation production under the assay conditions, whilst the fixation rate produced by 2,4-DAPG induction of NifA was 89% of WT culture rates. No background induction was seen in OPS3682 in the absence of 2,4-DAPG, whilst small amounts of background were seen from AHL strain OPS3681, though the difference was still highly significant compared to the induced state (*Figure 5.28*). The basal activity suggests some leaky expression of *nifA* from the P*lux* promoter.

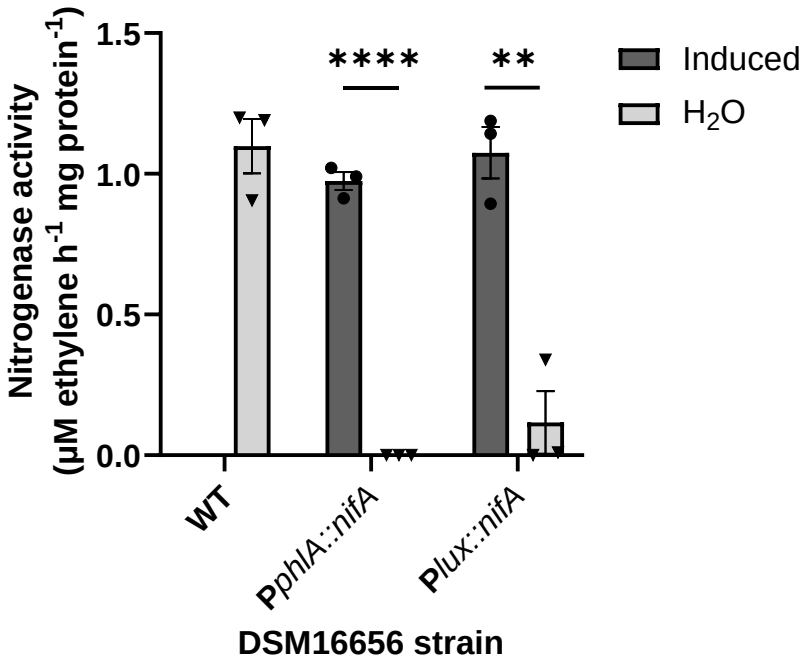


Figure 5.28 ARA at 3% oxygen of *E. radicincitans* strains P*lux::nifA* (OPS3681), and P*phlA::nifA* (OPS3682), alongside WT DSM16656. Cultures of OPS3681 were induced by 100nM 3-oxo-C6-HSL and OPS3682 by 20 µM 2,4-DAPG in UMS with 10 mM glucose and vitamin solution. (n = 3, ±SEM, independent Student's T-test, ** $p \leq 0.01$, **** $p \leq 0.0001$).

To further test the phenotype of the of *nifLA* deletion strain, the regulation of nitrogen fixation in response to ammonium was measured. Disruption of *nifL* can abolish inhibition of NifA not just in the presence of oxygen but also from regulation by exogenous fixed nitrogen. Expression of the closely related *Kosakonia sacchari nifA* from a heterologous promoter has been demonstrated to abolish regulation of *nif* gene expression by the PII regulatory cascade and decouple nitrogenase gene expression from the cellular nitrogen status[155]. To demonstrate this in our system, DSM16656 OPS3681 was induced for N_2 fixation in UMS with or without 10 mM ammonium chloride whilst inducing *nifA* expression with 100 µM 2,4-DAPG. WT DSM16656 alongside OPS3682 were grown overnight in LB followed by centrifugation and washing in UMS. Cultures were then resuspended in UMS with 10 mM glucose and vitamin solution to OD 0.5, with 10 mM ammonium chloride added to the high nitrogen assay

replicates. Cultures were incubated anaerobically for 12 hours in the presence of acetylene before measurement of N_2 fixation via ARA. The presence of ammonium in the assay media completely abolished N_2 fixation in WT DSM16656, however, the expression of *nifA* from the heterologous P*phlA* promoter restored the rate of N_2 fixation to comparable levels of the WT strain under high nitrogen assay conditions (*Figure 5.29*). This result demonstrates that the removal of transcriptional regulation of *nifA* in DSM16656 alleviates nitrogen repression of *nif* genes.

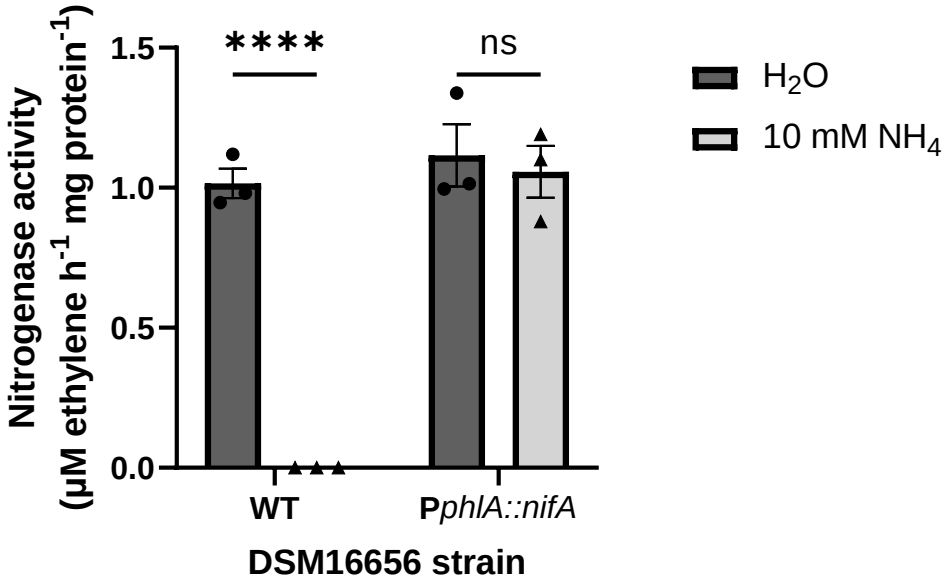


Figure 5.29 ARA at 3% oxygen of *E. radicincitans* strain P*phlA::nifA* OPS3682 and WT DSM16656 in the presence or absence of 10 mM ammonium chloride in UMS with 10 mM glucose and vitamin solution. OPS3682 was induced by 20 µM 2,4-DAPG. (n = 3, ±SEM, independent Student's T-tests, **** $p \leq 0.0001$).

To test the feasibility of the CL150 relay signals to control N_2 fixation in the DSM16656 inducible *nifA* strains developed, an *in vitro* ARA was performed to prove the concept before induction on engineered barley strains. To measure direct induction *nif* genes by CL150 derived relay signals, *E. radicincitans* AHL and 2,4-DAPG inducible *nifA* strains OPS3681 and

OPS3682, respectively, were grown overnight in LB alongside *S. meliloti* P*mocB::traI* (pOPS2100) and P*mocB::phlACB-PJ23106::phlDE* (pOPS2096) before diluting all cultures to OD_{600} 0.5 in UMS with 10 mM glucose, 10 mM ammonium chloride, and vitamin solution. *S. meliloti* and reporter strains were then mixed 50:50 in 4 mL with or without 10 µL SI. Mixed cultures were incubated for 6 hours before the addition of acetylene and measurement by GC after a further 12 hours (*Figure 5.30*).

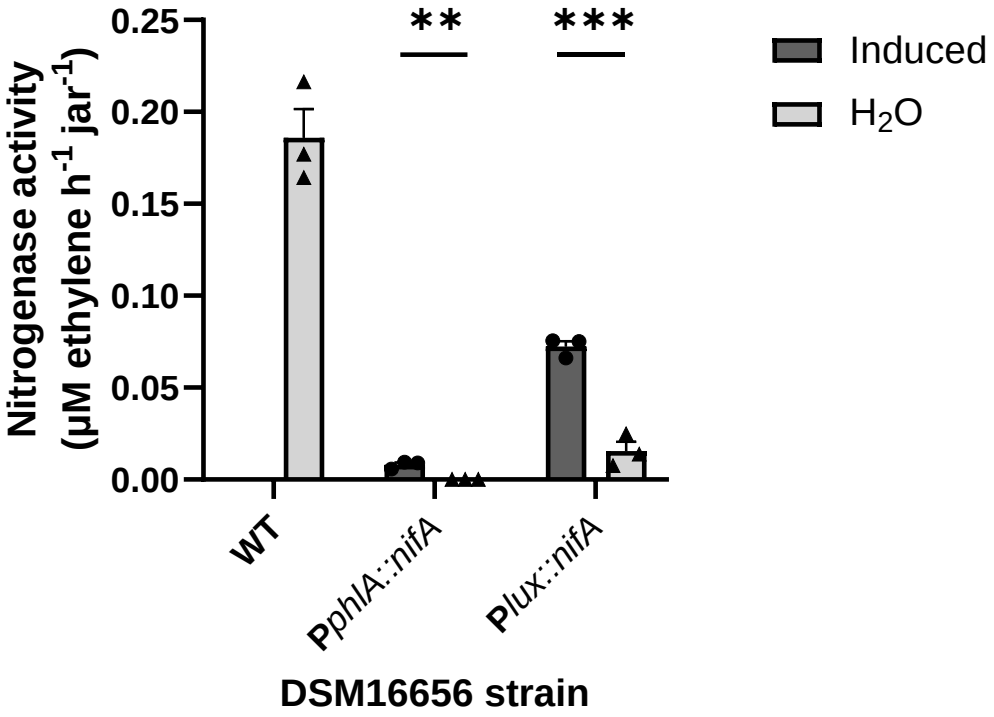


Figure 5.30 *In vitro* ARA at 3% oxygen of *E. radicincitans* strains P*lux::nifA* (OPS3681) incubated 1:1 with CL150 P*mocB::traI* (pOPS2100), and *E. radicincitans* P*phlA::nifA* (OPS3682) incubated with CL150 *PmocB::phlACB-PJ23106::phlDE* (pOPS2096), alongside WT DSM16656 in UMS with 10 mM glucose and vitamin solution. Cultures were induced by addition of 10 µM SI. (n = 3, ±SEM, independent Student's T-tests, ** $p \leq 0.01$, *** $p \leq 0.001$).

E. radicincitans strain OPS3681 showed a five-fold increase in N_2 fixation when induced with AHL produced by *S. meliloti* P*mocB::traI* in the presence of 10 μM SI. However, this rate of nitrogen fixation was just 39% of the nitrogen fixation rate measured in a solely DSM16656 WT culture (*Figure 5.30*). The difference in activity could possibly be due to incomplete induction of the entire *E. radicincitans* OPS3681 culture, as well as competition for carbon and nitrogen resources between DSM16656 and CL150. 2,4-DAPG production by CL150 was only able to produce a small amount of N_2 fixation by P*phlA::nifA* OPS3682, at only 5% of the relative mean fixation rate in WT cultures, though no leaky expression was observed.

5.4.9 *In Planta* Relay Induction of Nitrogen Fixation

To test the ability of the relay system to control N_2 fixation for PGP, an assay on *RhiP* barley plants was performed. The acetylene reduction assay was performed according to the method published by Haskett *et al,* 2021[249]. In short, a single colony of CL150 strains were grown on TY agar for 5 days before inoculation, whilst *E. radicincitans* strains were grown overnight in LB. The barley seeds were surface sterilized in 70% ethanol and 7% NaOCl followed by germination on 0.9% agar for 2 days. Germinated seeds were placed in a sterilized Schott bottle filled with 50 g of sand with 25 mL of rooting solution. To initiate the assay, CL150 relay strains were washed from agar slopes and resuspended to OD_{600} of 0.1 in UMS media before inoculation of 2 mL of bacterial solution around the seedling. The barley with CL150 were incubated for 5 days in a growth chamber with 23°C 16-hour light / 21°C 8-hour dark cycle before addition of 2 mL of OD_{600} 0.1 *E. radicincitans* NifA controller strains suspended in UMS. The plants were then incubated overnight before placing the Schott bottle in a 3% O_2 atmosphere growth cabinet for 1-2 hours. The bottles were then sealed and 10% of the headspace atmosphere replaced with acetylene, with measurements of ethylene production taken after 12 hours.

Incubation of CL150 P*mocB::traI* strain OPS3518 with P*lux::nifA* DSM16656 strain OPS3681 on *RhiP* producing barley gave a three-fold increase in N_2 fixation compared to the same strain on WT barley, demonstrating that bacterial relay signalling can function in response to plant derived rhizopine (*Figure 5.31*). The nitrogen fixation rate achieved by AHL relay signalling was 0.82µM ethylene h^{-1} $plant^{-1}$ which was 57% of the rate of WT *E. radicincitans* on *RhiP* barley, suggesting significant induction of P*lux::nifA* DSM16656 strains by the AHL relay system. However, the fixation rate achieved by P*phl::nifA* strain OPS3682 in response to the SI induced 2,4-DAPG signal was not significant from fixation on WT barley lines ($p = 0.15$), furthermore, ARA on *RhiP* barley by 2,4-DAPG signal was 12-fold lower than the AHL relay system, suggesting a problem with the production or viability of the 2,4-DAPG signal in the plant assay system (*Figure 5.31*).

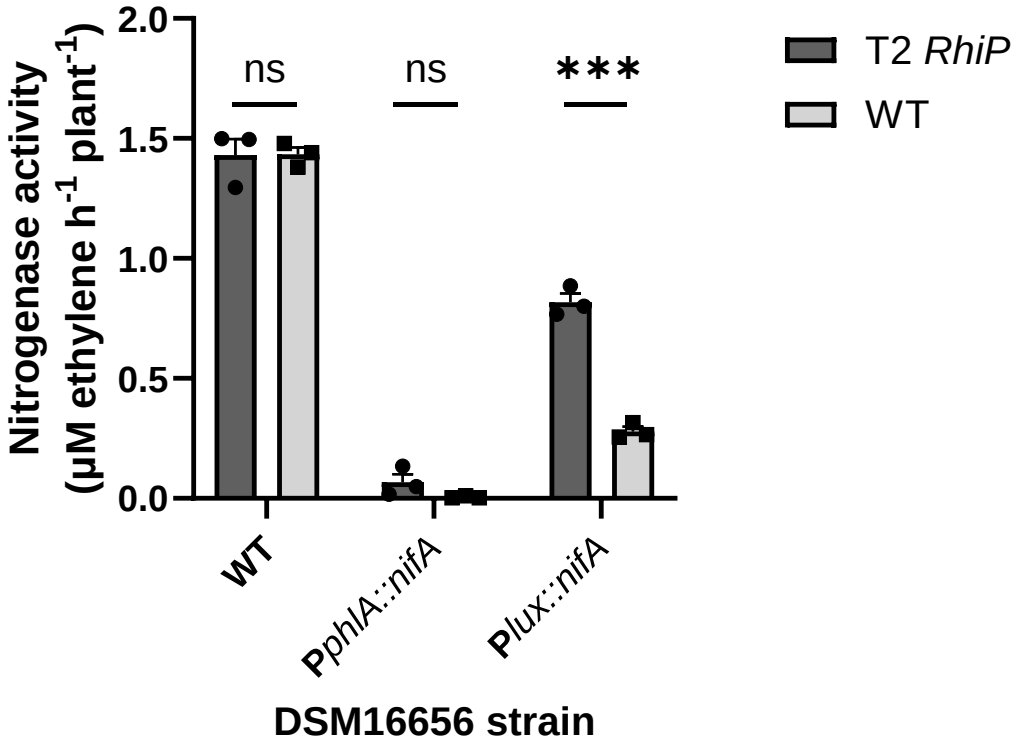


Figure 5.31 *In planta* ARA at 3% oxygen of *E. radicincitans* strains P*lux::nifA* (OPS3681) co-inoculated with CL150 P*mocB::traI* (OPS3518), and *E. radicincitans* P*phlA::nifA* (OPS3682) co-inoculated with CL150 *PmocB::phlACB-PJ23106::phlDE* (OPS3514), alongside WT DSM16656 on T2 *RhiP* barley. Nitrogen fixation was measured by replacing the headspace with 3% oxygen before addition of 10% acetylene and measurement of ethylene production 12 hours post induction. (n = 3, ±SEM, independent Student's T-tests, *** $p \leq 0.001$).

To measure the induction of *E. radicincitans* strains in the *RhiP* barley assay system, both P*lux::gfp* and P*phl::gfp* reporters, pOPS2155 and pOPS1537 respectively, were conjugated into constitutive mCherry marked *E. radicincitans* strain OPS2510. The mCherry marked strain was previously constructed in this lab via R6K integration of P*J23104::mCherry* downstream of *glmS*. The plasmid conjugation produced mCherry marked AHL inducible strain OPS3685 and 2,4-DAPG inducible OPS3692. These reporter strains allow the *E. radicincitans* populations to be isolated from the rhizosphere of *RhiP* barley via flow cytometry for measurement of the proportion of the bacterial population responding to each relay signal

on WT and *RhiP* barley. The assay was repeated as previously reported for ARA except with the addition of mCherry marked OPS3685 and OPS3692 to their respective relay assays at 5 days post inoculation with CL150 strains. After 24 hours barley seedlings were pulled up from the assay, the roots washed in PBS, and ground in a sterile pestle and mortar in 5 mL PBS to extract the root associated fraction of bacteria from the assay, which has been shown to have the greatest response to plant derived SI. The supernatant was passed through a sterile 40µm filter to clear growth substrate and diluted 10-fold in a 96 well plate for analysis of the bacterial population's mCherry and GFP fluorescence intensity. *E. radicincitans* populations were gated according to mCherry above 5000 arbitrary fluorescence units, and GFP fluorescence normalised to the 99th percentile fluorescence of reporter strains co-inoculated with WT CL150 on WT barley (*Figure 5.32*).

Analysis of *E. radicincitans* populations from AHL relay signal assays shows a mean population induction of 27.1% of P*lux::gfp* bacteria were induced by AHL relay signal in the root associated population of bacteria. In comparison, only 6.3% of the *E. radicincitans* population showed induction on WT barley plants, again demonstrating the leaky induction previously seen in both the P*mocB::traI and* P*lux* systems. However, the 4.3-fold difference in relative population induction reflects the differences in N_2 fixation seen with ARA in the *in planta* assay. In the case of the 2,4-DAPG relay signal, 4.9% of the *E. radicincitans* population responded to 2,4-DAPG induced on *RhiP* barley (*Figure 5.32*). This activity is greatly reduced compared to the AHL induced replicates and may be due to the decrease in sensitivity of the 2,4-DAPG responsive plasmid relative to the AHL reporter, or an issue with the stability of 2,4-DAPG biosynthesis and signal longevity over the course of the assay, leading to signal drift post inoculation and a reduced response.

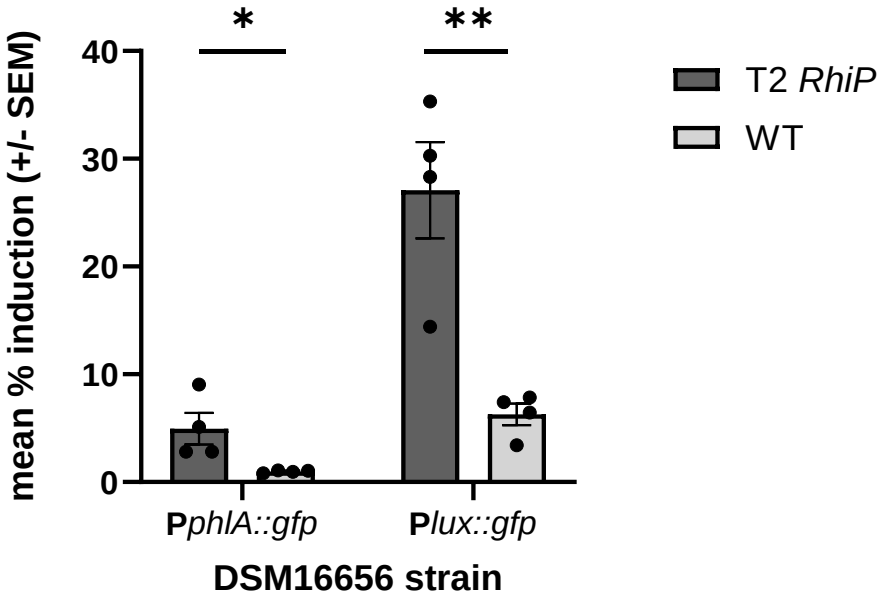


Figure 5.32 Percentage induction of *E. radicincitans* strains P*lux::nifA* (OPS3681) co-inoculated with CL150 P*mocB::traI* (OPS3518), and *E. radicincitans* P*phlA::nifA* (OPS3682) co-inoculated with CL150 *PmocB::phlACB-PJ23106::phlDE* (OPS3514) measured by flow cytometry of root associated fraction. *E. radicincitans* populations were gated according to mCherry above 5000 arbitrary fluorescence units, and GFP fluorescence normalised to the 99th percentile fluorescence of reporter strains on WT barley (n = 4, ±SEM, independent Student's T-tests, * $p \leq 0.05$, ** $p \leq 0.01$).

Flow cytometry analysis of mCherry marked *E. radicincitans* strains allows us to compare the relative populations of CL150 signal producers and DSM16656 signal responders in the assay. Analysis of the percentage of mCherry marked singlets within the overall sampled population gives a mean percentage of 32.03% (± 4.75) from the root associated bacterial population. As matching the relative growth rates of CL150 and DSM16656 is difficult, it would be interesting to titrate the relative populations of signaller and receiver to find the optimal ratio for each signal for maximal induction.

5.5 Discussion

5.5.1 Auxin Production

Nodule development requires local accumulation of auxin at the site of nodule initiation, where it stimulates cellular division of the root cortex and epricycle, and the phytohormone can improve host-plant response to stress[118,180,181]. To engineer auxin biosynthesis into rhizobacteria, the genes for the indole acetamide pathway from *P. savastanoi* tryptophan mono-oxygenase (*iaaM*) and indole-3-acetamide hydrolase (*iaaH*), were cloned into constitutive expression plasmids pOPS0666 and pOPS0667. The auxin biosynthesis plasmids were conjugated into *S. meliloti* and assayed for IAA production using Salkowski's reagent mixed with culture supernatant. Only an 8% increase in IAA production was seen with the auxin pBBR1 plasmid pOPS0666 relative to the WT, no significant difference was seen with the lower copy number RK2 plasmid pOPS0667. This difference was only present upon the addition of 1 mM tryptophan, a precursor of the indole acetamide pathway, to the growth media of *S. meliloti*. A large amount of IAA production was observed in the WT, which was expected as IAA production plays a role in the initiation of symbiosis in many rhizobia-plant host interactions[119,177].

To determine the relative production across rhizobacterial species, several strains were grown in TY and assayed for IAA secretion. In this assay *S. meliloti* CL150 produced low amounts of IAA relative to other rhizobia such as *A. brasilense, R. trifolii*, and *R. leguminosarum* 3841. It has been shown that indeterminate nodules formed on white clover (*Trifolium repens*) and pea (*Pisum sativum*) by these rhizobia is induced by an increase auxin response in early dividing cells of the nodule primordium mediated by the rhizobia[310,311]. It has also been shown that *S. meliloti* IAA production does mediate early nodule development[312], and an auxin overproduction strain of *S. meliloti* overexpressing *iaaM* and *tms2* has been shown

to enhance nodulation and lateral root growth in *Medicago* species[313]. The engineered *S. meliloti* IAA strain produced an auxin concentration of 1.2 nmol g^{-1} of nodule in *Medicago* to induce a stimulatory effect on nodulation[313], therefore, the increased IAA production in CL150 pOPS0666 may be too low to be detected by Salkowski reagent colourimetric assay[301]. Subsequently, the IAA biosynthesis plasmids were conjugated into *P. fluorescens* SBW25, which is more closely related to *P. savastanoi* from which the *iaaM* and *iaaH* genes were cloned from. The SBW25 IAA strain resulted in three-fold production of IAA relative to WT SBW25, which demonstrates the expressed IAA genes were functional.

Therefore, possible refactoring of the *iaaM* and *iaaH* genes for *S. meliloti* expression may improve the auxin biosynthesis in an engineered rhizobium. Furthermore, *S. meliloti* has been engineered to produce greater IAA by introducing an *iaaMtms2* construct into *S. meliloti* 1021 driven by the very strong *rolA* promoter from *A. rhizogenes*[80]. Therefore, a stronger promoter driving *iaaM* and *iaaH* in the construct used in this work may improve auxin production. Further evidence of engineering IAA production as a rational strategy is the expression of the *P. savastanoi* indole-3-acetimide genes in the non-PGP rhizobacterium *Cupriavidus pinatubonensis*[182]. The engineered strain was able to promote root and above-ground growth when inoculated on *Arabidopsis* plants. Furthermore, the indole-3-pyruvic acid pathway for IAA biosynthesis has been engineered in *E. coli*[300]. IAA production was facilitated in *E. coli* by the co-expression of genes required for the shikimate pathway of glucose conversion to L-tryptophan and then from tryptophan to IAA, thereby channelling metabolites from the carbon source for maximal IAA production. A similar strategy could be used in desired rhizobacterial strains to augment the IAA delivery to a host plant.

5.5.2 Relay Signalling

Haskett, *et al* demonstrated that rhizopine signalling, whilst a useful trans-kingdom signal, is not ubiquitous across bacterial taxa[248]. This limits the ability of host-dependent control of PGP mechanisms in more diverse rhizobacterial species. Control of PGP could be achieved by linking the biosynthesis of a more versatile secondary signalling molecule under SI control. Relay signalling would allow communication of the plant derived SI signal to PGP bacteria carrying a second cognate inducible system and therefore control of gene expression for PGP benefit for the host. Previous work has used diverse messenger signals to control promoters for *nif* gene expression in engineered rhizobacteria[99]. In this work, two messenger systems were developed to relay the rhizopine signal, a 2,4-DAPG dependent signal to control expression via de-repression of the PhlF regulator, and SI dependent AHL biosynthesis for induction of the LuxR positive regulator of gene expression.

For SI dependent production of 2,4-DAPG, the genes *phlACBD* required for the polyketide biosynthesis pathway, alongside *phlE* efflux protein gene from *Pseudomonas fluorescens* were cloned into constitutive expression (pOPS0910) and rhizopine inducible (pOPS0909) plasmids. Induction of an *E. coli gfp* biosensor by 2,4-DAPG from *S. meliloti* culture supernatant carrying the *phl* biosynthesis plasmids showed that constitutive expression produced ~300 μM 2,4-DAPG in culture. However, control of the rhizopine inducible *phl* plasmid pOPS0909 by the chromosomally integrated SI biosensor in *S. meliloti* strain OPS1864 produced only 20% of that amount. This reduction is likely due to the decreased sensitivity of the bacterium to the rhizopine signal, due to a single copy rhizopine biosensor, thereby leading to greatly reduced *phl* gene expression from P*mocB*. To improve SI induction of 2,4-DAPG biosynthesis, the *phl* genes were cloned under P*mocB* control on rhizopine plasmid biosensors pOPS1052 (medium copy) and pOPS0889 (high copy). These plasmids greatly increased the levels of 2,4-DAPG secretion by SI induced cultures, with the high copy pOPS2095 *phl*

plasmid producing 200 µM 2,4-DAPG upon induction by SI. 2,4-DAPG production was further improved by constitutive expression of *phlD*, responsible for the production of the 2,4-DAPG precursor monoacetylphloroglucinol (MAPG), and the efflux protein *phlE* in plasmid pOPS2096. This plasmid increased SI responsive 2,4-DAPG production in CL150 cultures to approximately 240 µM , which is comparative to the production from constitutive *phl* plasmid pOPS0910. However, the increase in expression of *phlD* and *phlE* permease led to greater 'leaky' production of 2,4-DAPG in the absence of SI, though induction produced a three-fold dynamic range in 2,4-DAPG secretion. The 2,4-DAPG relay signal was capable of direct induction of *E. coli* P*phl::luxCDABE* expression in the presence of SI when the cultures were grown adjacently, demonstrating its ability to act as a relay signal in bacterial consortia.

A second SI inducible relay signal was developed based on the 3-oxo-hexanoyl-HSL quorum sensing molecule. AHL-based quorum sensing signals are well studied in rhizobia where they are able to regulate bacterial gene expression within a population[54]. QS signals in rhizobia are involved in switching from free living to symbiotic lifestyles by inducing motility, *nod* signalling, and genes required for host colonisation such as EPS production[288,289,314]. To use AHLs as a rhizopine relay signal, the LuxR/LuxI family AHL system from *Mesorhizobium* WSM1497 was cloned. This QS system regulates transfer of *sym* genes on integrative and conjugative genetic elements[315]. The AHL system is composed of the AHL regulator TraR, and AHL synthase TraI1. In this system TraI produces 3-oxo-C6-HSL which is bound by the LuxR homologue TraR, this complex promotes expression of *traI* in a positive feedback loop to produce more 3-oxo-C6-HSL. The AHL system was cloned into rhizopine biosensor pOPS1889 and tested for induction of 3-oxo-C6-HSL induction by co-culturing with the *E. coli luxR*-P*lux::luxCDABE* biosensor. The TraR-TraI system under control of P*mocB* in plasmid pOPS2097 exhibited a constitutive phenotype. Leaky induction from P*mocB* coupled with the TraR positive induction of TraI lead to constant production of AHL, even in the absence of the

SI. To mitigate this, a weak RBS was placed in-front of the *traR* regulator in plasmid pOPS2099. This construct produced a 15% decrease in the level of AHL secreted by CL150 strains without SI induction. Although this is significant, it does not give enough dynamic range in a signal to regulate effective PGP in recipient bacteria. Therefore, just the AHL synthase *traI* gene was placed under control of P*mocB* in the rhizopine inducible plasmid pOPS2100. Whilst this resulted in a 45% decrease in SI induced production of AHL, it reduced AHL production in the off state, giving a 7.5-fold dynamic range in induced signal production.

The SI inducible relay signals were used to control nitrogen fixation in the gamma-proteobacterium cereal endophyte *E. radicincitans*, which is incapable of responding to SI. To control N_2 fixation, a Δ*nifLA* strain was produced by homologous recombination with a PK19mobSacB suicide vector. *E. radicincitans* is predicted to have a similar regulatory mechanism of nitrogen fixation to other gammaproteobacterial diazotrophs, whereby the *nifLA* operon directly regulates the *nif* gene cluster through the transcriptional activator NifA, and nitrogen and oxygen dependent repression of NifA by NifL. This mode of regulation was supported by N-insensitive fixation in *E. radicincitans ΔnifLA* carrying *phlF*-P*phlA*::*nifA* plasmid pOPS2154 which produced WT rates of nitrogen fixation in the presence of 10 mM ammonium chloride.

Both *S. meliloti* produced 2,4-DAPG and AHL relay signals were assayed for their ability to regulated *nif* gene expression in *E. radicincitans* by regulating *nifA* expression. *In vitro, E. radicincitans* strain OPS3681 with plasmid P*lux::nifA* co-cultured with CL150 carrying P*mocB::traI* plasmid pOPS2100 was capable of 39% the rate of WT nitrogen fixation. The reduction in N_2 fixation rate likely reflects the reduction in resource availability due to the presence of *S. meliloti* in the culture. However, induction of *E. radicincitans* P*phlA::nifA* by the 2,4-DAPG relay signal produced by *S. meliloti* pOPS2096 produced only 5% of WT *E. radicincitans* rates of N_2 fixation. The decreased rate may be due to the reduced sensitivity of

P*phlA* induction relative to AHL induction of P*lux*, or the potential inhibition of nitrogen fixation by 2,4-DAPG. Furthermore, co-inoculation of the CL150 relay strain and *E. radicincitans* inducible *nifA* strains was performed on *RhiP* barley plants. Incubation of CL150 P*mocB::traI* strain OPS3518 with P*lux::nifA* DSM16656 strain OPS3681 on SI producing barley achieved a nitrogen fixation rate of 57% the rate of WT *E. radicincitans* on *RhiP* barley which suggests significant induction of P*lux::nifA* DSM16656 strains in the AHL relay system. There was a three-fold difference in N_2 fixation in the AHL system between *RhiP* and WT barley lines demonstrating the rate of nitrogen fixation was achieved by SI specific induction of CL150 AHL biosynthesis. More importantly, the rate of N_2 fixation achieved by *E. radicincitans* induction on *RhiP* barley was 225-fold greater than the rates achieved by SI control of *A. caulinodans* N_2 fixation when colonising T2 *RhiP* barley roots. However, no significant nitrogen fixation was seen in the 2,4-DAPG induction system on *RhiP* barley relative to WT lines, suggesting a problem with 2,4-DAPG signal viability over the 6-day course of the assay. It is possible that 2,4-DAPG production was limited by resource competition or downregulated by the cell via the silencing of SI transport genes due to the metabolic burden of production, as seen with pOPS0910 growth, or that cells use hydrolases to degrade 2,4-DAPG to the less toxic monoacetylphloroglucinol (MAPG) and acetate[316].

The induction of *E. radicincitans* populations by the SI dependent relay signals *in planta* was measured by flow cytometry analysis. Results showed 27% of the *E. radicincitans* population isolated from the root-associated fraction was induced by the AHL relay signal, whilst 2,4-DAPG responsive populations showed a small 4.9% difference in percentage induction between WT and *RhiP* lines. This result demonstrates the feasibility of plant control of more diverse PGP bacteria through microbial consortia. Furthermore, the AHL relay signal acted as an amplifier of plant derived SI, as *E. radicincitans* induction in this assay was 2-fold higher than CL150 induction in populations isolated from the roots of *RhiP* barley (chapter 4

figure 4.9) Despite the inability of *S. meliloti* produced 2,4-DAPG to induce nitrogen fixation in *E. radicincitans* in the rhizosphere under these assay conditions, it would be interesting to measure the production of 2,4-DAPG by *S. meliloti* at intervals post inoculation to see if the levels secreted fluctuated. Furthermore, if μM quantities of 2,4-DAPG were produced on *RhiP* barley it could be tested for the capability of antagonising colonisation of phytopathogenic fungi, such as *Rhizoctonia solani,* in the rhizosphere. Thereby contributing to PGP by biocontrol in a diverse rhizosphere.

A key problem with this method for controlling N_2 fixation is balancing the abundance of messenger and receiver strains in the rhizosphere of target host plants. The *in planta* assay performed in this work resulted in a 2:1 ratio of *S. meliloti* messenger to inducible nitrogen fixing *E. radicincitans*. Titrating the relative abundances of each bacterial population would allow the development of the optimum ratio of messenger and receiver bacteria and would be dependent on signal strength and sensitivity. One strategy to balance bacterial populations would be to implement synthetic auxotrophy through the deletion of one or more essential metabolic genes in the responding bacterium making it dependent on an exogenous metabolite for growth and survival, the supply of which could be controlled by the relay. Alternatively, strains could also be linked by the AHL relay signal inducing expression of an essential gene in the recipient bacterium, linking the growth of the strain to the concentration of the relay signal. The target bacterial population could also be enriched by utilisation of a sole specific carbon or nitrogen source, such as opine metabolism in *Agrobacterium* populations, or *moc* catabolism genes for use of the plant derived rhizopine signal. Furthermore, the use of more specific and heterologous signalling molecules could reduce the crosstalk and noise which would undoubtably be present in a diverse rhizosphere. The abundance of QS systems in both symbiotic and pathogenic plant bacterium highlights the need for the synthetic bacteria-to-bacteria signalling circuitry for control of gene expression in the rhizosphere. Novel

heterologous signals with limited noise and crosstalk would allow the extension of bacteria-bacteria control of further PGP mechanisms, such as phosphate solubilisation and phytohormone production, to diverse communities of engineered inoculants.

Chapter 6

Conclusion

6.1 Overview

NifA is a key regulator of nitrogen fixation in diazotrophic proteobacteria. It allows these bacteria to integrate environmental signals to regulate *nif* gene expression in order to balance the benefit of N_2 fixation and assimilation in nitrogen deficient environments with the cellular fitness costs of protein synthesis and ATP consumption by the nitrogenase complex. NifA acts as an enhancer binding protein and requires the alternative sigma-factor σ^{54} to bind the enhancer elements upstream of *nif* genes, whereby the initiation complex hydrolyses ATP to catalyse a conformational change to the open promoter complex and gene transcription[84]. The activation of σ^{54}-dependent *nif* gene transcription was used in this work to demonstrate control nitrogen fixation in diverse alpha and gamma proteobacteria.

6.2 Control of NifA for Free Living Nitrogen Fixation

The versatile cereal endophyte *A. caulinodans* was used to engineer control of free living nitrogen fixation via control with the rhizopine trans-kingdom signal. The rhizopine signal was previously shown to be able to control gene expression in bacteria colonising the surface of transgenic cereal roots[201,227]. The bacterial biosensor responding to rhizopine was tuned for increased sensitivity by the inclusion of *myo*-inositol transporters from *R. leguminosarum* Rlv3841, allowing for the expression of transmembrane components in *A. caulinodans* for the transport of rhizopine into the bacterial cytoplasm. Co-expression of the ATP-binding ABC transporters IntBC with the *Rhizobium* solute binding protein MocB improved the sensitivity of the rhizopine biosensor by 10^3-fold, with induction of *gfp* expression by rhizopine *in vitro* seen at concentrations as low as 1µM. To use the rhizopine signal to control nitrogen fixation in *A. caulinodans*, *nifA-rpoN* were placed under control of the rhizopine responsive *PmocB* promoter in the biosensor plasmid pOPS0889. This controller

plasmid (pOPS1122) was initially shown to specifically induce *nif* genes in response to exogenous rhizopine using a P*nifH::mCherry* reporter. In *A. caulinodans*, cellular oxygen and nitrogen status environmental signals are integrated via transcriptional regulation of *nifA* to provide control of *nif* gene expression in free living diazotrophs[129-131]. In our SI controller plasmid system in a Δ*nifA* background, this transcriptional regulation of *nifA* is abolished and replaced by the SI input. However, the removal of transcriptional regulation was unable to completely abolish fixed nitrogen repression of *nif* gene induction in our assay.

A. caulinodans is unusual for a symbiotic rhizobium as its N_2 fixation is inactivated under high nitrogen conditions in laboratory cultures, whilst most symbiotic rhizobia only fix N_2 in nodules where oxygen predominantly regulates NifA[46,83,91]. The regulation of N_2 fixation suggests post-transcriptional regulation of the NifA protein by the cellular nitrogen sensing machinery in *A. caulinodans*. NifA regulation was partially overcome by introducing a mutation in the GAF domain of NifA, identified in nitrogen insensitive *R. capsulatus* mutants[99]. The GAF domain appears to be responsible for regulation across diazotrophic species, but its sequence is poorly conserved allowing for variation in the signal regulation of NifA activity across species[86,130,211,317]. In free living diazotrophs such as *K. pneumoniae* and *A. vinelandii*, the role of nitrogen regulation is often performed by the GAF domain of NifA interacting with NifL in a two component regulatory system[149,152,154]. However, rhizobial diazotrophs lack this regulatory system and the NifA GAF domain is regulated directly by alternative signalling cascades[82,135]. The oxygen sensitivity of rhizobial NifA has been demonstrated in multiple strains and is mediated by a cysteine-rich motif in an inter-domain linker between the AAA+ and HTH domains[45]. The GAF domain is not required for NifA activity, with a reported strong increase in NifA activity upon deletion of the GAF domain in *S. meliloti,* whilst partial deletion resulted in a non-functional protein[82,318]. Conversely, deleting the GAF domain in Rlv3841

abolishes activity, possibly due to protein misfolding or inability to escape deactivation when this domain is absent[45].

It is possible that the GAF domain of *A. caulinodans* NifA is post-transcriptionally repressed by PII proteins as in *A. brasilense*[209,317], or is directly binds 2-oxoglutarate under low nitrogen conditions to prevent inactivation as in *A. vinelandii*[154]. It is also feasible that the remaining nitrogen regulation seen in *A. caulinodans* $nifA_{l94Q/D95Q}$-*rpoN* strains is due to direct regulation of the nitrogenase complex. Nevertheless, the nitrogenase activity of the $nifA_{L94Q/D95Q}$-*rpoN* SI controller measured by ARA demonstrated robust control of nitrogen fixation in a cereal endophyte.

Despite nitrogen insensitive control of N_2 fixation in engineered *A. caulinodans*, this strain did not secrete excess fixed nitrogen into the growth media. This result is often seen in free living diazotrophs which exhibit mechanisms that couple nitrogen fixation with assimilation of ammonia by the cellular GS enzyme[136]. In diazotrophs the cellular nitrogen status is sensed by the PII signal-transduction proteins which are modified by uridylylation under nitrogen limitation[137]. In turn, PII proteins regulate the activity of GlnE which subsequently activates GS by deadenylylation[252]. *A. caulinodans* possesses two PII proteins GlnB and Glnk, it has been reported than insertional inactivation of these proteins leads to ammonia secretion due to repression of GS activity due to persistent adenylylation[129]. We aimed to reproduce this mutant in our *A. caulinodans* NifA controller strain via markerless deletion[254].

We were able to select for single gene deletions of either PII protein, however we were unable to obtain double deletions, due to a lethal phenotype which prevent cell growth. Furthermore, single gene mutants were unable to secrete ammonia under nitrogen fixing conditions, suggesting both PII proteins are capable of regulating GlnE independently. In the

reported double deletion strain, the insertional antibiotic resistance cassette was targeted to the 3' end of the *glnB* gene and therefore left enough of the 5' reading frame intact to retain some ability related to its essential cellular function[129]. Similar attempts to simultaneously delete *glnB* and *glnK* PII proteins in *R. capsulatus* were unsuccessful but single insertional inactivation resulted in nitrogen insensitive *nifA* transcription and nitrogenase activity[138]. In *R. capsulatus,* the post-translational ammonium inhibition of NifA activity was completely abolished in PII mutant strains[138], suggesting GAF domain repression by PII proteins which may also be present in *A. caulinodans*.

6.3 Control of Symbiotic Nitrogen Fixation

To achieve control of nitrogen fixation in a symbiotic rhizobium, refactored *nif* clusters from *K. oxytoca* were used induced in *E. coli* and *S. meliloti.* Nitrogen fixation was only achieved with refactored cluster v2.1, with no activity seen with cluster v3.2. The nitrogenase rate was significantly lower in rhizobium compared to *E. coli* which reflects reported data[99]. The benefit of refactored clusters is the removal of stringent host regulatory control on *nif* genes and nitrogenase activity due to the burden of N_2 fixation on metabolic resources. Native regulation is removed by the recoding and reorganisation of genes alongside the use of characterised synthetic RBS and promoters[205,267]. A controller is used to regulate the expression of the *nif* gene cluster, in our case an IPTG inducible T7 RNAP controller, which showed good activity in *Sinorhizobium*. Transfer of the v2.1 cluster resulted in nitrogenase activity in *E. coli* and low levels of activity in *Sinorhizobium*. Transfer of *nif* clusters between species can be affected due to reduced activity of promoters and terminators affecting transcription rates and RBS sites producing different translation rates in alternate hosts, impacting the stoichiometry of genes[99]. RNA-sequencing could be performed on the induced v2.1 cluster in *S. meliloti* to ascertain if this was the case, however given the minimal nitrogenase activity produced by this strain, this is not a viable strategy to pursue control of nitrogen fixation.

In this work, no activity was seen with cluster v3.2, which contained optimised promoters and terminators for more stringent expression control in rhizobia and WT *K. oxytoca* operon structure to preserve expression ratios[99]. This result was likely due to altered promoter sequences which likely abolished the expression ratios of key *nif* operons. More research is required to understand the complex nature of heterologous genes cluster activity between strains. Comparison of the transfer of 10 native clusters from diverse bacteria into non-diazotrophic bacteria *E. coli, P. protegens* Pf-5*, and R. sp* IRBG74 was tested. The activity of *nif* clusters was found to be greater between more closely related bacterial classes[99]. The conserved activity was likely due to similar underlying genetic parts (promoters, RBSs, and terminators) and codon usage of the genes preserving the expression rates of the *nif* genes between species. Some *nif* clusters from versatile diazotrophs such as *A. caulinodans* were too large, containing 64 Kb across 76 genes and possessing complex regulation, to be able to engineer for transfer between strains[99]. Therefore, the use of controllers to regulate native *nif* is a more successful strategy for widespread control of nitrogen fixation.

The strategy of rhizopine control of *nifA* expression for engineered nitrogen fixation demonstrated in *A. caulinodans* was tested in the symbiotic rhizobia *S. meliloti*. A range of rhizopine biosensor plasmids was tested in *S. meliloti*. Induction of the *intBC-mocB-mocR* plasmid pOPS0889 was higher in *S. meliloti* CL150 relative to *A. caulinodans,* likely due to the origin of biosensor components from the closely related *S. meliloti* L5-30 strain. Strains carrying this plasmid were found to be growth defective when induced by SI, possibly due to toxic accumulation of SI within the cell by the inositol transporters IntBC. To reduce the dosage of IntBC transporters, an integrated biosensor was tested in the CL150 chassis. However, induction by SI of the integrated biosensor could only be detected via complementation with a plasmid encoding *mocR*-P*mocB::gfp*. The integrated system reduced the growth defect of rhizopine induction but was not stable over the course of an *in planta* assay on barley *RhiP*

lines. It is possible that the dosage of transporters was too low to transport the reduced concentrations of rhizopine exuded from the roots of SI barley, or that the single gene copy of integrated transporters was susceptible to mutagenesis over the course of the assay.

To further decrease the growth defect of SI induction, a weak *S. meliloti* promoter *PrpoD* was used to drive *intBC* expression. The resulting biosensor pOPS1998 was less sensitive to rhizopine across the concentrations tested and still exhibited reduced growth upon induction. A balance of SI sensitivity and reduced metabolic burden on the cell was found by encoding the SI perception genes on a lower copy RK2 plasmid. Strains carrying this plasmid showed the most stable SI perception, with the highest mean percentage induction on the roots of *RhiP* barley. The low-copy biosensor plasmid was therefore chosen as the best strategy to control *nifA* for nitrogen fixation in the nodule environment.

We were unable to demonstrate control of nitrogen fixation in the nodules of *RhiP Medicago* by SI induction of *nifA*. Despite higher percentage induction of SI perception by bacteria in the nodule environment vs root colonisation, the overall induction was lower, suggesting reduced concentrations of SI in the nodule environment. It was shown that T1 *RhiP* barley produced seven-fold more rhizopine than T2 lines[201], therefore repeating the assay with T1 *RhiP Medicago* may elicit a greater response in SI responsive bacteria and enable *nif* gene induction. To improve engineered nitrogen fixation for synthetic symbiosis, it will likely be required to further engineer the production and sensing of SI in the plant host and corresponding bacterial partner so that it is less of a metabolic burden. Further optimisation would improve the signal stability and increase the percentage of bacterial cells occupying roots that perceive rhizopine and activate gene expression for N_2 fixation.

6.4 Control of Diverse Rhizobacteria for PGP

The range of rhizopine signalling to bacteria is limited to species in the alphaproteobacterial class of bacteria. This limitation appears to be due to incompatibility of the mocR/mocB promoters in more diverse strains and the inability of MocR to promote transcription from P*mocB* with the RNA polymerase or sigma factors found outside of this class. This was demonstrated by the expression of MocR from synthetic *E. coli* promoters which still failed to promote gene expression from *PmocB*[248]. However, there is a wealth of diverse diazotrophs outside of this class of bacteria which may be engineered to promote plant growth[35,156]. Of particular interest are gammaproteobacterial, which includes several species with the ability to fix nitrogen under free living conditions[155,319]. Controllers for nitrogen fixation have been constructed with agriculturally relevant sensors and are a key point at which the synthetic regulation of a gene cluster can be altered[99].

In this work, two well-characterised messenger systems were developed to relay the rhizopine signal, a phloroglucinol based 2,4-DAPG dependent signal to control expression via derepression of the PhlF regulator, and SI dependent AHL biosynthesis for induction of LuxR positive regulation of gene expression. These two signals are already used by bacteria in the rhizosphere for competitive colonisation and plant-growth promotion[288,304]. For SI dependent production of 2,4-DAPG, the genes *phlACBD* required for the polyketide biosynthesis pathway, alongside *phlE* efflux protein gene from *Pseudomonas fluorescens* were cloned under the P*mocB* promoter of high and low copy rhizopine biosensor plasmids. These plasmids enabled production of μM concentrations of 2,4-DAPG production in response to rhizopine, which was further increased by constitutive expression of *phlD*, responsible for the production of the 2,4-DAPG precursor monoacetylphloroglucinol (MAPG), and the efflux protein *phlE* in plasmid pOPS2906. This signal enabled direct induction of *E. coli* grown in co-culture with *S. meliloti* producing 2,4-DAPG in response to an initial rhizopine signal.

A second signal based on the *Mesorhizobium* AHL quorum signal was also developed. Cloning of the AHL regulator *traR*, and AHL synthase gene *traI1* under P*mocB* control resulted in constitutive AHL production due to the leaky expression exhibited by the rhizopine biosensor and the autoinduction loop produced by TraR promotion of P*traI1* transcription. The constitutive activity was mitigated by placing the *traI* AHL synthase gene under control of P*mocB*.

To demonstrate the ability of these alternate signals to regulate *nif* expression in bacteria outside of the alpha subgroup of proteobacteria, the cereal endophyte *E. radicincitans* was chosen as a candidate for engineering[140]. A closely related strain *K. sacchari* was isolated from the plant-associated fraction of corn roots[155]. It was found to be a robust associative diazotroph amenable to genetic engineering for BNF and ammonia release. Work to derepress the complex host regulation of free living nitrogen fixation in gammaproteobacteria, such as *E. radicincitans,* has focused on manipulating the two-component negative (NifL) and positive (NifA) regulators that integrate environmental signals and control *nif* transcription[153,214,253].

In many gammaproteobacteria, the activity of NifA is not intrinsically oxygen or nitrogen responsive, and instead NifL modulates NifA activity directly in response to the cellular status of these signals[320]. We capitalised on this approach to produce a nitrogen insensitive *E. radicincitans* strain via the deletion of the native *nifLA* operon and replaced *nif* regulation with an inducible *nifA* controller. This controller decouples nitrogenase biosynthesis from the nitrogen responsive PII protein regulatory cascade[148,149]. Synthetic control of *nifA* expression in a Δ*nifLA* strain resulted in WT rates of BNF in the presence of fixed nitrogen. Furthermore, an engineered *E. radicincitans* Δ*nifLA* strain carrying AHL inducible *nifA* plasmid pOPS2100 to produce ~40% of WT rates of nitrogen fixation *in vitro* when co-cultured with SI induced *S. meliloti* carrying *PmocB::traI.* The reduction in the rate of nitrogen fixation

is likely due to the sharing of resources between the two bacterial strains reducing the metabolic efficiency and therefore the cellular energy available for BNF.

Nitrogen fixation with the 2,4-DAPG signalling relay system in *E. radicincitans* was eight-fold lower than AHL induction, possibly reflecting the reduced sensitivity of the *phlF*-P*phlA* biosensor, or the instability of the signal. However, the nitrogen insensitive nitrogenase activity seen with AHL induction may be sufficient to generate an excess of fixed nitrogen within the cell which cannot be assimilated by GS, leading to passive excretion of ammonium out of the cell, which has been observed in *nifL* deletions in *A. vinelandii*[153,214]. We were unable to test this hypothesis during this work, but it would enhance the viability of this engineered system for BNF. If this was not the case, AHL induction of *nifA* could be coupled with expression of a truncated GlnE protein lacking its adenylyl-removing (AR) domain, which would only be capable of adenylylating GS, thereby limiting ammonium assimilation by the cell and promoting excretion of fixed nitrogen[252,321].

Incubation of CL150 P*mocB::traI* strain OPS3518 with P*lux::nifA* DSM16656 strain OPS3681 on SI producing barley achieved a nitrogen fixation rate of 57% relative to WT *E. radicincitans* on *RhiP* barley, with a three-fold dynamic range in resulting fixation between WT and *RhiP* systems. Flow cytometry analysis of AHL induced *E. radicincitans* populations on *RhiP barley* showed 27% of the population responded to the plant induced QS signal, over two-fold greater than the induction of CL150 SI responsive biosensors colonising the root of *RhiP* barley, demonstrating that the AHL relay signal successfully amplified the initial host plant derived rhizopine signal for greater induction of bacteria and increased N_2 fixation. Therefore, this system could be used to control diverse communities of PGP rhizobia in the rhizosphere of transgenic cereals for PGP.

The 2,4-DAPG signal was unable to induce effective nitrogen fixation in *E. radicincitans* on barley plants. This result could possibly be due to the toxicity of phloroglucinol production in the cell which could be caused by reduced levels of malonyl-CoA, a precursor for fatty acid synthesis and elongation, or due to the accumulation of toxic intermediate metabolites and/or end products that are debilitating to the bacteria over the course of the *in planta* assay[295]. Alternatively, the production of 2,4-DAPG could be too metabolically costly for the bacterium whilst competing for resources with another bacterial strain. If the sustainable production of 2,4-DAPG could be improved, possibly by metabolic engineering to increase the malonyl-CoA pool, the effect of 2,4-DAPG for control of phytopathogens could be tested[294,297,298]. The *phlACBD* operon was expressed in the diazotrophic wheat endophyte *Pseudomonas* species WS5 which was subsequently able to control fungal pathogens *Magnaporthe oryzae* and *R. solani* to promote cereal growth via host protection[298].

In plant associated bacteria, QS signals coordinate gene expression for virulence, colonisation, and symbiosis, as well as promote the defence-priming of plant hosts[289]. Colonising bacteria form biofilms on the host plant root surface, regulated by QS systems which promote biosynthesis of EPS, lipopolysaccharides, and bacterial motility for host colonisation[265,289]. The ubiquity of the QS signalling process has evolved quorum-quenching bacterially produced enzymes, alongside the ability of plants to alter the QS-regulated biofilm formation of plant-associated bacteria via AHL-mimicking molecules, suggesting that plants can promote or inhibit root colonisation dependent upon the bacterial strain[292,322,323]. Further studies would contribute to a better understanding of plant-bacteria communities and the identification of more specific signalling molecules. These in turn could be used to promote the formation of diverse PGP bacterial root communities and be used to regulate further beneficial processes such as phosphate solubilisation, iron-scavenging, phytohormone biosynthesis, and rhizoremediation[156,166].

6.5 Future Direction

Much work is still needed to understand the regulation of *A. caulinodans nifA* in both symbiotic and free living conditions. It is still unclear how NifA and nitrogenase activity are regulated by cells under these conditions. NtrC has been reported to activate NifA targets, and may be responsible for some of the effect of ammonium chloride concentration on reporter activity that we observed[132]. Despite the diversity in diazotrophic transcriptional regulation of *nifA*, the ability of NifA to regulate gene targets appears to be better conserved. This conserved activity allows the possibility of heterologous expression for cross-species complementation that can bypass native regulation[82,83,324,325]. Using NifA from another diazotroph to regulate nitrogen fixation in *A. caulinodans* represents a potentially useful strategy to further overcome regulation for engineered nitrogen fixation but remains to be explored. Another approach to overcome the remaining regulation could be to create a library of NifA variants, produced through combinatorial assembly of domains from different NifA proteins, which could then be screened for activity using the assays developed in this work.

Despite the success of controlling *E. radicincitans* N_2 fixation in the rhizosphere of an engineered cereal, fixation is only beneficial in an agricultural setting if the bacterial partner can release significant quantities of ammonia to the plant for host assimilation[25]. Therefore, regulation of *nif* gene transcription by host specific signals must also be connected to strategies for the release of fixed nitrogen. Although it has shown to be possible to promote ammonia release through induced suppression of the bacterial NH_3^+ assimilation pathways, such engineering methods can severely impact host physiology and therefore the viability and fitness of an inoculant in field settings[155,214,326]. The cellular burden could be mitigated by the hosting of engineered bacteria in a protected environment, such as pseudo-nodules which can be induced on cereal roots[183,185]. However, due to the complexity of legume nodule regulation and development, we are far from achieving true nodulating symbioses in cereals[26,95].

Flow cytometry analysis of *S. meliloti* SI biosensors colonising *RhiP* barley roots and *Medicago* nodules showed only a limited fraction of the bacterial population were responding to the exuded rhizopine signalling, thereby limiting the effectiveness of this strategy to regulate effective nitrogen fixation for BNF. This result suggests that increasing rhizopine production by *RhiP* plants could promote greater population perception of SI and greater PGP response by engineered strains. However, increased production would have to be coupled with further optimisation of the rhizopine biosensor and tuning the expression of genetic components for SI transport and induction to balance the sensitivity of SI perception with minimal growth repression for sustainable and robust signalling *in planta*[327]. Use of the rhizopine signal as a method of target population enrichment also remains to be tested. Maintaining a sufficiently dense population of an engineered bacterium in the root-associated fraction would be critical for any engineered plant-microbe interaction, and would be detrimentally impacted by the expression of PGP traits[328]. An enrichment process, similar to the evolution of opine biosynthesis and catabolism in pathogenic *Agrobacterium* species, could be developed using the *moc* locus for the catabolism of rhizopine[221,222,228]. Engineering strain utilisation of rhizopines as a source of C and N would promote a competitive advantage for colonisation of the host plant by that strain[229,231].

Use of rhizobia with plant-controlled induction of specific AHLs could further enhance the beneficial effects of colonising bacteria and enlarge the PGP effects of bacterial inoculants beyond the capabilities of once particular strain. Expanding the host range of rhizopine would require significant engineering of compatible RNA-polymerases or the selection of engineered small-molecule aptamers capable of binding rhizopine for integration into synthetic riboswitches for gene induction[329]. Alternatively, further rhizosphere specific signals regulating genes could be identified as alternative plant-bacterial signals. Crop exudation profiles could be studied to identify specific root exudate compounds that may induce bacterial

gene expression. Conversely, bacterial RNA-sequence studies could be performed on different plant-bacterial colonisation assays to identify upregulated bacterial genes in the rhizosphere and their cognate signal-inducible promoters. However, an efficient trans-kingdom signal is only as good as the phenotypic traits it regulates in bacterial partners. Considering the strong selection pressure for rapid growth and colonisation in the rhizosphere[330], further mechanisms of genetic regulation, particularly by O_2, N, and C, which promote strain fitness over any benefits to colonised plant hosts, need to be identified and overcome. Therefore, the developments made in this work represent a small step towards the establishment of efficient synthetic symbiosis between engineered cereals and bacterial inoculants required for long-term sustainable agriculture.

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