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REVIEW ARTICLE

Engineering rhizobacteria for sustainable agriculture

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Abstract

Exploitation of plant growth promoting (PGP) rhizobacteria (PGPR) as crop inoculants could propel sustainable intensification of agriculture to feed our rapidly growing population. However, field performance of PGPR is typically inconsistent due to suboptimal rhizosphere colonisation and persistence in foreign soils, promiscuous host-specificity, and in some cases, the existence of undesirable genetic regulation that has evolved to repress PGP traits. While the genetics underlying these problems remain largely unresolved, molecular mechanisms of PGP have been elucidated in rigorous detail. Engineering and subsequent transfer of PGP traits into selected efficacious rhizobacterial isolates or entire bacterial rhizosphere communities now offers a powerful strategy to generate improved PGPR that are tailored for agricultural use. Through harnessing of synthetic plant-to-bacteria signalling, attempts are currently underway to establish exclusive coupling of plant-bacteria interactions in the field, which will be crucial to optimise efficacy and establish biocontainment of engineered PGPR. This review explores the many ecological and biotechnical facets of this research.

Introduction

To meet the food demands of a near 10-billion strong human population projected to exist on Earth by 2050, agricultural productivity must increase by up to 70% [1–3]. This onerous challenge must be met without expansion of arable land, and using current or lower inputs of environmentally deleterious agrochemicals (fertilisers and pesticides) or we risk further loss of natural ecosystems and depletion of rock phosphate reserves [2, 4]. Plant growth promoting (PGP) rhizobacteria (PGPR) offer many services to plants that could substitute the roles of agrochemicals to boost primary food production [5]. Exploitation of PGPR as inoculants of high-yielding cereals such as wheat, maize and rice, that constitute 42.5% of human caloric intake [6], has specifically emerged as a promising strategy to drive the sustainable intensification of agriculture.

While some PGPR have already been commercialised and can significantly improve crop yield [7–10], field performance of PGPR is typically inconsistent [11, 12], in-part due to underdeveloped inoculation technology which falls outside of the scope of this review, but also as a consequence of the following issues (Fig. 1): Firstly, colonisation of the rhizosphere – the root-soil interface where nutrient exchange occurs between bacteria and plants – may be suboptimal due to competition with resident microbes that have become well-adapted to the soil conditions over years of natural selection. Secondly, while plants exert some control over the composition of the root associated microbiome (rhizomicrobiome), colonisation by PGPR typically lacks stringent host-specificity [13]. As a result, wild or invasive plant species may benefit from the services of PGPR, enhancing their ability to compete with the target crop for resources. Lastly, some PGPR have evolved agriculturally undesirable modes of genetic regulation to repress PGP traits when they are not beneficial to the bacteria, helping them to conserve energy and resources [14, 15], but consequently rendering them of reduced benefit to the plant.

While some highly competitive and stress-tolerant cereal rhizosphere colonising bacteria have been isolated from different soil types, the key genetic aspects underlying their efficaciousness remain to be defined [16]. In contrast, many mechanisms of PGP including nitrogen (N)-fixation, phosphate (P)-solubilisation, phytohormone biosynthesis, rhizoremediation of xenobiotic pollutants and biocontrol of pathogens have been elucidated in fine enough detail to be genetically engineered. Transfer of these traits on domesticated mobile genetic elements (MGEs) into selected rhizobacterial "chassis" or entire populations can be used to

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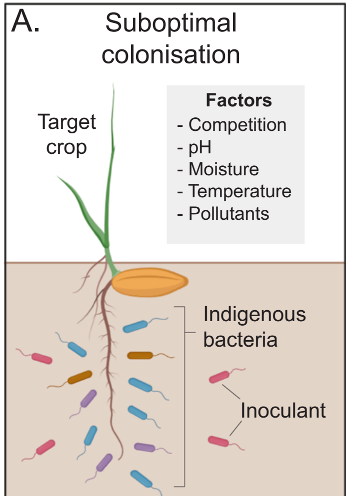

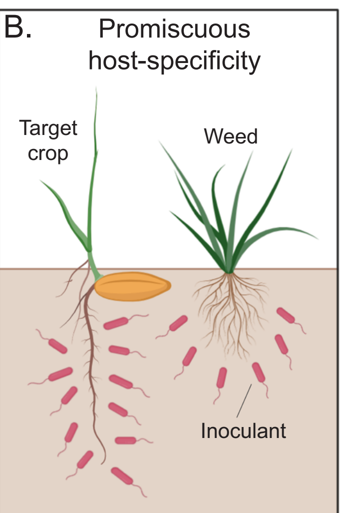

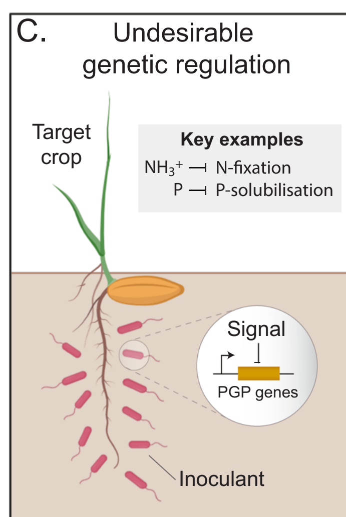

Fig. 1 Limitations of natural PGPR. The benefits of PGPR in agriculture are restricted by three factors. **a** Inoculant bacteria may fail to colonise the rhizosphere of target crops to exert their beneficial effects due to competition with resilient resident microbes that may have become well-adapted to the soil conditions over years of evolutionary selection. Abiotic stresses may also impact negatively on persistence in the bulk-soil. **b** Although plants can exert some control over the structure of their rhizomicrobiome, there is no stringent host-specificity for bacterial colonisation of plant roots. Thus, inoculant PGPR may provide their beneficial services to non-target invasive species, creating competition for the target plant. **c** Some PGPR have evolved agriculturally undesirable modes of genetic regulation that repress expression and/or activity of PGP traits when conditions are not conducive for the bacteria (See Fig. 2 for additional information). Such regulation can assist the bacteria in conservation of energy and resources but renders the bacteria of little benefit to plants.

tailor effective PGPR with desirable traits for agricultural use in specific soil types. By utilising recently developed synthetic host-specific plant-to-bacteria signalling [17], it has become possible to establish exclusive coupling of plant-bacteria interactions in the field, which will subsequently will allow us to begin optimising various aspects of engineered PGPR ecology, including enrichment, PGP efficacy and biocontainment. In this review, we highlight ecological and biological limitations of natural PGPR in agriculture and discuss how genetic engineering and synthetic biology are being applied to develop improved PGPR tailored for agricultural use.

Limitations of natural PGPR

Suboptimal rhizosphere colonisation and persistence in foreign soils

In comparison to the oligotrophic bulk soil, the rhizosphere is a highly favourable environment for microbial proliferation due to the secretion of root exudates rich in sugars, organic acids and amino acids [18, 19]. PGPR must first colonise the rhizosphere to exert their beneficial effects on plants (Box 1). Most of the natural rhizosphere microbiota is inherited vertically from the bulk-soil and forms a complex community which matures and becomes structurally stable after only 2 weeks [20]. PGPR used to inoculate crops are typically applied directly to the seed but despite their initial proximity to the developing root, PGPR must compete for colonisation of the rhizosphere with resilient resident microbiota that may have become well-adapted to the soil conditions over years of evolutionary selection (Fig. 1a).

Competitive rhizobacteria may utilise adaptive structural or metabolic traits that enhance nutrient acquisition or provide tolerance to abiotic stresses such as pH, drought, salinity and soil chemistry [21]. Environmental pH is the main determinant of microbiome selection at the phylum level. Acidic soils are dominated by Acidobacteria, whereas

Box 1. Bacterial colonisation of the rhizosphere

Bacterial colonisation of the rhizosphere first involves a dispersal phase, where the bacteria recognise primary metabolites in root exudates and migrate towards the rhizoplane (root surface) by chemotaxis [19]. Epiphytic bacteria attach to the rhizoplane leading to the establishment of micro-colonies or biofilms. Subsequently, endophytic bacteria colonise the root endosphere, entering plant tissues through natural wounds or fissures formed at the base of lateral roots. Endophytes can alternatively force entry into the intercellular environment by secretion of cellulases or other enzymes that degrade the plant cell wall [19, 143]. The genetics of root colonisation are immensely complex and are therefore generally probed using "omic" approaches [16]. Transposon insertion sequencing experiments suggest that over one-hundred amino acid catabolism, stress adaptation, detoxification, signal transduction and transport genes contribute to the fitness of the epiphyte *P. aeruginosa* PGPR2 during colonisation of maize roots [144]. Motility, polysaccharide and other general surface attachment factors are likely also important for this process [145].

Proteobacteria colonise soils with neutral-pH and phyla are evenly distributed in high-pH soils [22]. Competitive PGPR may additionally harbour microbial weapons such as type IV, VI and VII secretion systems, contact-dependent growth inhibition, nanotubes for delivery of toxins and outer membrane exchange. Remarkably, bacteria have even mastered phage weaponry such as tailocins to disrupt opponent membranes and can harbour active phage in their genomes for targeted bacterial killing [23].

Although very few genetic determinants of rhizosphere colonisation, persistence and competition under stress have been elucidated, bioprospecting for efficacious rhizosphere colonising bacteria in different soil types has been fruitful. For example, drought tolerant PGPR that stimulate seedling germination and growth of *Setaria italica* (foxtail millet) have been isolated from semi-arid regions in northeast China [24], whereas many halotolerant PGPR have been described [25]. Utilising competitive PGPR that are well-adapted to their environment is crucial to achieve optimal colonisation of target crops, but ultimately limits the scope of PGP traits available for agricultural exploitation.

Promiscuous host-specificity

Establishment of intimate N-fixing endosymbioses between rhizobia and legumes involves a complex dialogue of molecular communication utilising plant-derived flavonoids and bacterial-derived Nod-factors to achieve a "lock and key" type strategy for partner-specific infection [26]. In contrast, rhizosphere colonisation and subsequent PGP by both epiphytic and endophytic rhizobacteria lack active mechanisms for stringent partner-specificity (Fig. 1b), which could be problematic in the field due to promiscuous colonisation and growth promotion of wild or invasive plant species which compete with target crops for light, resources and space, negatively impacting yield [27]. Plants can exert some control over the composition of the rhizomicrobiome by producing root exudates with distinct compositions of metabolites that stimulate or repress microbial proliferation [28]. In a key study where natural blends of phytochemicals derived from *Arabidopsis* root exudates were added to soil in lieu of the plant, pyrosequencing of 16s rRNA revealed that phenolic compounds dramatically increase the number of unique operational taxonomic units (OTUs), whereas sugars and amino acids had a similar, but less profound effect [29]. Analysis of the rhizomicrobiome structure of an *Arabidopsis* ABC transporter mutant, which exhibits increased export of phenolics and decreased export of sugars, has further revealed selective stimulation of PGPR relative to a wild-type control [30]. In addition to the above-mentioned phytochemicals, camalexin, coumarins, triterpenes, benzoxazinoids, and in some cases, their degradation products, have been shown to influence rhizomicrobiome structure [31, 32]. Importantly, the composition of an individual plants root exudates are not static, but rather, are dynamically influenced by the plant developmental stage, presence of abiotic stresses and by the rhizomicrobiome itself [28]. Thus, it is difficult to predict root exudate composition in the field.

Undesirable regulation of PGP traits

In the context of PGP, many processes beneficial to the plant are energetically costly to the bacterium. As such, PGPR have evolved tight regulatory systems to control expression of PGP genes and activity of PGP traits in response to environmental or internal conditions, enabling conservation of energy and resources (Fig. 1c). The existence of this agriculturally undesirable regulation means that some PGPR may perform sub-optimally in the field. Epiphytic and endophytic diazotrophic rhizobacteria that reduce chemically inert atmospheric N_2 gas into NH_3^+ for plant assimilation are prime examples (Fig. 2). Most catalyse N-fixation via molybdenum-dependent nitrogenases that are highly sensitive to O_2 and may require expression of up to 35 *nif* and accessory genes for their assembly and function [33]. N-fixation is extremely energy intensive, consuming 16 mol ATP per mol N_2 fixed in vitro. However, the in vivo cost of N-fixation may reach as high as 42 mol ATP per mol N_2 fixed when factoring nitrogenase expression, O_2 protection mechanisms and metabolic rerouting of electron allocation [34–36]. To reduce energy consumption, diazotrophs have evolved strict multi-layered regulation of nitrogenase, primarily in response to NH_3^+, O_2 and in some species, C [14]. O_2 and C regulation ensure nitrogenase is only expressed when conditions are conducive (i.e. O_2, high energy) preventing futile expression. In contrast, NH_3^+-regulation, which has become integrated into global bacterial N-metabolism, provides negative feedback for N-fixation preventing production of excess NH_3^+ that is not required for assimilation [14]. Because of this feedback regulation, natural diazotrophic bacteria secrete little of their fixed N for assimilation by plants.

Phosphate-solubilising bacteria represent a second key example to illustrate the problem of undesirable genetic regulation in PGPR. Bacterial phosphatase and phytase enzymes that release available phosphorus (P) from various sources of organic matter are typically regulated via the Pho regulon [15] which permits expression of the genes only under P limitation [37–39]. Because plants likely become starved of P before bacteria, this regulation may prevent phosphate-solubilising bacteria reaching their full potential for phosphatase/phytase expression, and thus PGP in the field. Considering the strong selective pressure fast growing bacteria in the rhizosphere [18], it seems is likely that many modes of gene regulation which favour

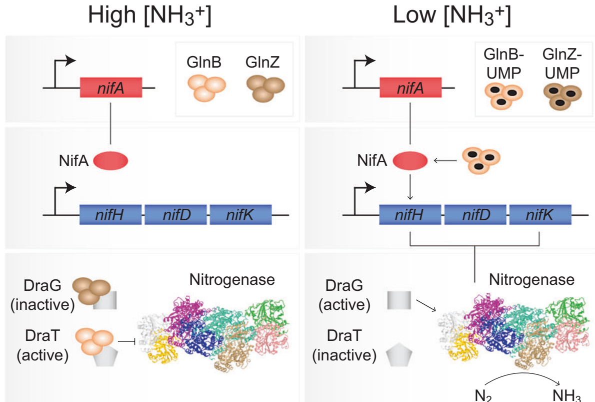

Fig. 2 Examples of multi-layered NH_3^+-dependent repression of N-fixation. NH_3^+-dependent negative feedback regulation of N-fixation is ubiquitous in diazotrophic bacteria and involves diverse multi-layered strategies that primarily target the "master regulator" of N-fixation NifA, which acts in association with the sigma factor σ^{54} to drive expression of a large suite of nitrogenase (*nif*) and accessory genes. For simplicity, σ^{54} is not shown here, and only the structural nitrogenase genes *nifHDK* are shown as induced by NifA. In all bacteria, the N-status of the cell is sensed via GlnD, which under low N-conditions, uridylylates one or more global N-regulatory P_{II} protein(s) (GlnK, GlnB and GlnZ in the depicted systems) [14]. **a** In *Klebsiella pneumoniae*, uridylylated GlnK phosphorylates NtrB leading to subsequent phosphorylation of NtrC which in turn activates transcription of *nifLA* [141]. NifL is able to bind NifA and inhibit its activity, but is itself inactivated by binding to uridylylated GlnK under low NH_3^+ conditions. **b** In *Azospirillum brasilense*, *nifA* expression is constiutitve but the NifA protein requires binding to uridylylated GlnB for activation [142]. Nitrogenase activity is additionally regulated via the DraT-DraG system in this strain [142]. Under high NH_3^+ conditions, DraT binds to de-uridylylated GlnB and inactivates nitrogenase through ADP-ribosylation, whereas DraG binds to de-uridylylated GlnZ and is sequested to the membrane. Under low NH_3^+ conditions, nor DraT or DraG bind their cognate P_{II} protein and DraG subsequently re-activates nitrogenase by reversal of ADP-ribosylation.

preservation of bacterial fitness over plant fitness remain to be identified.

Engineering and transfer of PGP traits

In recent decades, a plethora of gene clusters conveying distinct PGP traits have been assembled on domesticated broad-host-range MGEs for transfer into rhizobacterial chassis' [40]. By mobilising PGP traits into stress-resistant, competitive, and efficacious root-colonising rhizobacteria (Box 2), we can tailor strains with desirable PGP traits for specific soil types. Transfer of such gene clusters may additionally suffice to overcome natural modes of undesirable genetic regulation where essential factors are absent in the recipient strain [41], though the genomes of recipients occasionally require further metabolic tweaking to optimise functionality. In this section, we provide an overview of some the key PGP traits that have been genetically engineered (Table 1), successfully transferred and, in some cases, optimised in rhizobacteria.

N-fixation

Current agricultural productivity is largely dependent on supplementation of crops with N that has been synthesised through the Haber-Bosch process [42]. The production of this nitrogenous fertiliser alone consumes 1–2% of global annual energy supply and accounts for 1.5% of global carbon emissions [43], whereas excess application of nitrogenous fertiliser can result in unintended contamination

Box 2. Strategies for transfer of PGP traits

Engineering of PGPR can be achieved using bottom-up or top-down strategies [135]. The traditional bottom-up strategy first involves isolation of rhizobacteria followed by introduction of PGP gene clusters on broad-host-range plasmids through conjugation or phage-transduction. Relative to conjugative plasmids that are readily lost from bacterial populations, integrative and conjugative plasmid systems such as mini-Tn*7* [146] and chassis-independent recombinase-assisted genome engineering (CRAGE) [147] offer the advantage of long-term stability and are the gold standard for transfer. The more recently defined top-down strategy for transfer of PGP genes does not require isolation of single bacteria but can be used to introduce foreign genes into undomesticated populations in situ via a conjugal donor strain. To illustrate this technology, a miniaturised mobilisable derivative of the integrative and conjugative element ICE*Bs1* has been mobilised into diverse bacteria inoculated into sterile soil via a donor strain *B. subtilis* XPORT [148]. in situ conjugative transfer of extrachromosomal and integrative BHR plasmids into the mammalian gut microbiota was also recently achieved in a similar fashion [149]. The ability to transfer PGP into bacterial populations in situ will be instrumental in driving high-throughput generation of repurposed PGPR and has sparked the emergence of microbiome engineering as a defined field of study.

Table 1 Key engineered PGP traits.

Trait	Recipient strain(s)	Transferred gene(s)	Function/comments	PGP phenotype	Source(s)
N-fixation	*Escherichia coli*	14*K. oxytoca nif* genes expressed as 5 cleavable polyproteins	"Fuse-and-cleave" virus-derived polyprotein strategy used to achieve optimised stoichiometric expression of nitrogenase genes for N-fixation.	NT	[53]
	E. coli	9 *nif* genes and *pfoAB-fldA* from *P. polymyxa* and *nifSU* from *K. oxytoca*	Co-expression of *nif* genes with an electron transporter (*pfoAB-fldA*) and Fe–S cluster assembly genes (*nifSU*) for markedly improved N-fixation.	NT	[47]
	E. coli	*K. oxytoca* refactored *nif* cluster v1.0	Initial refactoring of *K. oxytoca nif* genes for N-fixation in *E. coli*.	NT	[51]
	E. coli	*K. oxytoca* refactored *nif* cluster v2.1	Significantly increased N-fixation effectiveness through combinatorial reorganisation of v1.0 cluster.	NT	[52]
	E. coli	*K. oxytoca* native *nif* genes	First demonstration of cloned *nif* gene transfer to convey N-fixation.	NT	[44, 150]
	Pseudomomas protogens Pf-5	*P. stutzeri* native *nif* cluster	First demonstration of N-secretion and PGP by a *nif* gene recipient.	Various aspects of *Arabidopsis*, alfalfa, tall fescue and wheat growth.	[41]
	Rhizobium sp IRBG74	*K. oxytoca* refactored *nif* cluster v3.2	First demonstration of N-fixation with gamma-proteobacterial *nif* genes in an alpha-proteobacterium.	NT	[45]
	Rhizobium sp IRBG74	Native *nif* genes from *R. sphaeroides*	Fixed significantly more N then *K. oxytoca* refactored *nif* cluster v3.2 when expressed in same background.	NT	[45]
	Synechocystis sp. PCC 6803	24 *nif* and accessory genes and *hupSLW* from *Cyanothece sp.*	Co-expression of *nif* genes and hydrogenase for improved N-fixation under microaerobic conditions.	NT	[48]
P-solubilisation	*P. fluorescens*	*S. elongatus ppc*	Overexpression of phosphenol pyruvate carboxykinase (*ppc*) enhanced production of organic acids and improved P-solubilisation.	NT	[59]
	P. fluorescens	*E. coli gltA* and *citC*	Expression of citrate synthase (*citA*) and exporter (*gltA*) improved citrate production and P-solubilisation.	NT	[60]
	P. putida, P. simiae and Ralstionia sp.	82 biochemically diverse phytases	41/185 strains expressing a phytase solubilised phytate in liquid culture.	12/14 strains tested promoted increased biomass and rosette area of *Arabidopsis*.	[63]
IAA biosynthesis	*Cupriavidus pinatubonensis*	*P. savastanoi iaaH* and *iaaM*	Biosynthesis of IAA using indole-3-acetamide biosynthesis pathway under quorum-sensing control.	Increased lateral root number, root length, fresh weight and rosette area of *Arabidopsis*.	[68]
	E. coli	*E. cloacae ipdC, E. coli aspC and Ustilago maydis iad1*	De novo biosynthesis of IAA using Indole-3-pyruvic acid pathway.	NT	[70]
	E. coli	*Saccharomyces cerevisiae aro8* and *kdc* and *E. coli aldH*	De novo biosynthesis of IAA using indole-3-pyruvic acid pathway. Several modifications made to shikimate acid pathway to boost substrate (L-tryptophan) production from glucose.	NT	[69]

Table 1 (continued)

Trait	Recipient strain(s)	Transferred gene(s)	Function/comments	PGP phenotype	Source(s)
Naringenin biosynthesis	*E. coli*	*Rhodotorula glutinis TAL, Petroselinum crispum 4CL, Petunia X hybrida CHS, Medicago sativa CHI, Rhizobium trifolii matBC*	*De-novo* biosynthesis of naringenin following modular optimisation of an entirely synthetic pathway.	NT	[71]
Biocontrol	*P. fluorescens* SBW 25	*P. synxantha* 2-79 *phzABCDEFG*	Biosynthesis of the antifungal phenazine-1-carboxylic acid (PCA). Shown to have no impact on arbuscule mycorrhizal establishment of field margin plants.	Improved various aspects of growth for pea, wheat and sugarbeat infected with *Pythium ultimum*.	[82, 83]
	P. fluorescens strains BL914 and BL922	*P. fluorescens prnABCD*	Biosynthesis of the broad-spectrum antifungal pyrrolnitrin.	Reduced presence and severity of *Rhizoctonia solani*-induced lesions on cotton.	[79, 80]
	Pseudomonas sp. WS5	*Pseudomonas* sp. G22 and *P. protegens* Pf-5 *phlDACB*	Biosynthesis of the antigungal 2,4-diacetylphloroglucinol (DAPG).	Improved various aspects of growth for rice and sorghum infected with *Magnaporthe oryzae* B157 and *R. solani*.	[81]
Rhizoremediation	*Azospirillum brasilense* Cd	*acdS*	Anhydotetracycline-regulated expression of ACC-deaminase (*acdS*).	Increased root length in tomato and canola.	[94, 151]
	Burkholderia cepacia L.S.2.4	*Burkholderia cepacia* G4 plasmid pTOM	Transfer of plasmid pTOM conveyed toluene mono-oxygenase activity to degrade toluene.	Increased biomass of yellow lupin grown under toluene stress.	[96]
	Enterobacter sp. E5 and *Kosakonia* sp. S1	*P. syringae inaK* fused to *P. fluorescens acdS*	Ice nucleation protein (*inaK*) fused to *acdS* facilitated cell-surface expression of ACC-deaminase.	Increased root and shoot length of rice under salt stress.	[95]
	P. putida 06909	Synthetic EC20	Expression of synthetic phytochelatin (EC20) to improve intracellular Cd^{2+} binding.	Increased biomass of sunflower grown under Cd^{2+} stress.	[99]
	Ralstonia. eutropha MTB and *P. aeruginosa* Pse	Chimeric Mtb (Mouse MT and *Neisseria gonorrhoea igA*)	Chimeric metallothionein (Mtb) transported to the cell surface and adsorbed cadmium from the environment.	Increased biomass and chlorophyll content of *Nicotiana bentamiana* and green pea, respectively, grown under Cd^{2+} stress.	[97, 98]

NT not tested.

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of ecosystems with severe cumulative effects [42, 43]. Exploitation of bacterial N-fixation offers a highly sustainable and efficient alternative to the use of N-fertilisers in agriculture.

For decades, engineering PGP traits has focussed primarily on N-fixation which has seen rampant progression since the transfer of the *Klebisiella oxytoca* M5a1 *nif* cluster into *Escherichia coli* in 1972 [44]. A single study recently demonstrated transfer of 12 *nif* gene clusters between 15 diverse bacterial species [45]. It has become clear that the effectiveness of N-fixation is strongly influenced by the genotype of the *nif* recipient bacteria. Transfer of a single 9-gene *nif* operon from the cereal epiphyte *Paenibacillus polymyxa* WLY78 into *E. coli* initially resulted in 10% nitrogenase activity compared to the wild-type donor strain [46], but this could be boosted to 50% by co-expression of the *P. polymyxa* electron transporter genes *pfoAB-fldA* and the *K. oxytoca* nitrogenase Fe-S cluster assembly genes *nifSU* [47]. In a separate study, transfer of 24 N-fixation genes from the photosynthetic cyanobacterium *Cyanothece* sp. into *Synechocystis* sp. PCC 6803 conveyed 30% nitrogenase activity in anoxic conditions compared to the donor strain. Although N-fixation was strongly inhibited under microaerobic conditions, co-expression of a hydrogenase gene cluster *hupSLW* increased N-fixation 2–6-fold presumably by improving the oxygen tolerance of nitrogenase [48]. These studies highlight the necessity for broad-scope engineering of nitrogenase accessory genes and oxygen tolerance mechanisms to achieve optimal N-fixation in naturally non-diazotrophic bacteria.

Overcoming natural NH_3^+-dependent feedback regulation of N-fixation represents one of the principal challenges for the generation of strains that readily release fixed N for assimilation by plants [14]. Interestingly, while *Pseudomonas stutzeri* A1505 does not fix N in the presence of NH_3^+, transfer of the *nif* genes into the cereal endophyte *P. protogens* Pf-5 enabled the recipient to undergo NH_3^+-insensitive N-fixation. The recipient released fixed N into the environment and promoted various aspects of *Arabidopsis*, alfalfa, tall fescue, wheat and maize growth under N-limiting gnotobiotic conditions [41, 49]. Similar NH_3^+-insensitive regulation of N-fixation was observed for 3/5 alternative *Pseudomonas* species receiving the *P. stutzeri nif* genes, whereas NH_3^+-regulation was preserved in the remaining two species. Inter-species transfer of *nif* genes can abolish native modes of regulation due to the absence of regulatory factors in the recipient, but remarkably, N-regulation was preserved when the *P. stutzeri nif* genes were transferred into *E. coli* [50]. Strong evidence suggested that GlnG, an *E. coli* homologue of NtrC (Fig. 2b), was repressing expression of the *nif* master regulatory *nifLA* genes in the presence of NH_3^+. Transfer of *nif* clusters between more distantly related bacteria, for example from gamma-proteobacteria to alpha-proteobacteria and vice versa, typically results in the generation of non-fixing strains due to transcriptional incompatibility [45]. Thus, the origin of *nif* clusters and the recipient must be carefully considered to produce compatible and effective N-fixing strains.

Modular refactoring of *nif* clusters under artificial transcriptional control offers a controlled strategy to circumvent native regulation but perplexingly requires accurate reproduction of Nif protein stoichiometry for optimal nitrogenase assembly and function. Although, initial refactoring of the *K. oxytoca nif* cluster under transcriptional control of a T7-polymerase in *E. coli* gave only 10% of nitrogenase activity relative to the donor strain [51], this was boosted to 57% by introducing a variant *nif* cluster v2.1 which had been optimised by combinatorial optimisation of modules [52]. Surprisingly, transfer of *nif* cluster v2.1 into the cereal endophytes *P. protogens* Pf-5 and *Rhizobium* sp. IRBG74 did not convey these bacteria with the capacity for N-fixation [45] but subsequent introduction of a v3.2 *nif* cluster with natural operonic structure and IRGB74-optimised genetic components conveyed low rates of N-fixation in these strains [45]. A "fuse-and-cleave" virus-derived polyprotein strategy has also been used to refactor 14 *K. oxytoca nif* genes into five translational reading frames that convey N-fixation in *E. coli* that is 72% as effective as *K. oxytoca* [53]. This strategy has the potential to revolutionise engineering of complex PGP traits that require precise reproduction of protein stoichiometry.

Ultimately, if engineered N-fixing rhizobacteria are to be utilised for agriculture, they must release significant quantities of fixed N for plant assimilation. Considering the plethora of research involving transfer of *nif* clusters, it seems inconceivable that *P. protogens* Pf-5 carrying the *P. stutzeri nif* genes remains the only *nif* recipient strain that has been shown to secrete N and promote plant growth [41, 49]. For *nif* recipients that fail to secrete fixed N, it is possible to force secretion through conditional suppression of the NH_3^+ assimilation pathways, albeit such modifications can severely impact on viability and fitness, rendering strains poorly suited to field conditions [14, 54]. The development of less debilitating strategies to force N-secretion will be invaluable for the development of efficacious N-fixing bacteria for agriculture.

Phosphate solubilisation

Although phosphorus (P) is abundant in soils in both organic and inorganic forms, only around 0.1% of the total P content is available to plants due to poor solubility [55]. Akin to the "nitrogen crisis", current agricultural productivity is also heavily reliant on supplementation of crops with P-fertilisers, but these are in finite supply and

ecosystem scale disturbance to P cycling is environmentally deleterious [4, 56, 57]. Phosphate-solubilising bacteria release metabolically inaccessible P in soil and therefore, could be agriculturally exploited to reduce dependency on P-fertilisers and improve the efficiency of P use within agro-ecosystems [57].

The principal mechanism for inorganic phosphate (Pi) solubilisation in natural bacteria involves lowering of soil pH by secretion of strong organic acids [58]. Engineering strategies have attempted to replicate this. Overexpression of *Synechococcus elongatus* PCC 6301 phosphoenolpyruvate carboxylase (*ppc*) in *P. fluorescens* was shown to enhance glucose metabolism, increasing production of gluconate, pyruvate and acetate, which mildly improved Pi solubilisation [59]. An artificial citrate operon containing an *E. coli* derived NADH-insensitive citrate synthase (*gltA1*) and citrate transporter (*citC*) has also been successfully cloned and integrated into the genomes of six PGP *P. fluorescens* strains, elevating secretion of citric and gluconic acids and increasing solubilisation of mineral phosphate [60]. Although the above-mentioned bacteria mobilise P under laboratory conditions, artificially enhanced organic acid production and secretion demands substantial inputs of C, likely exceeding what is available in most rhizospheres. Therefore, the efficacy of P mobilisation by these bacteria would presumably be suboptimal in the field.

Organic phosphate also represents a crucial P reservoir in the soil, typically accounting for 20–60% of total P [61], but reaching as high as 90% in high organic matter soils [62]. Bacteria can produce diverse enzymes, such as alkaline phosphatases, acid phosphatases, phytases, phosphonatases, nucleases and phosphodiesterases, which release P from various sources of organic matter [15, 57]. A recent study used a combinatorial synthetic biology-based approach to generate 82 biochemically diverse phytase enzymes which were integrated into the genomes of *P. putida*, *P. simiae* and *Ralstonia* sp. strains [63]. Of 185 strains generated, 41 mobilised P from phytate in liquid culture and 12/14 tested promoted increased biomass and rosette size of *Arabidopsis* grown under gnotobiotic conditions with phytate as a sole source of P. Crucially, enzymatic release of P offers a far less carbon-intensive strategy to release phosphates in the soil for plant assimilation, but it remains to be discerned whether experimental results can be replicated in the field.

Auxin and naringenin biosynthesis

The ability of PGPR to influence plant development through production of phytohormones and other developmental effectors can enhance biomass, provide tolerance to abiotic stresses and improve root colonisation by resident rhizobacteria [64–67]. A *P. savastanoi* derived indole-3-acetamide biosynthesis pathway facilitating production of the auxin phytohormone indole-3-acetic acid (IAA) from L-tryptophan has been expressed in *Cupriavidus pinatubonensis* [68]. When inoculated onto *Arabidopsis*, the resulting strain increased lateral root number, root length, fresh weight, and rosette area. Alternative synthetic indole-3-pyruvic acid pathways for IAA biosynthesis from L-tryptophan have also been engineered into *E. coli* [69, 70]. Crucially, these pathways rely on the presence of large intracellular quantities of the precursor L-tryptophan that may not be present in the rhizosphere. To circumvent this issue, several genetic modifications have been made to an *E. coli* strain carrying IAA biosynthesis genes that increase flux through the shikimate pathway and drive increased production of L-tryptophan from glucose [69]. The resulting strain produced IAA when grown on glucose as a sole carbon source.

Engineering of biosynthesis pathways to produce the plant-derived flavone naringenin in *E. coli* has received much attention in recent times mainly owing to the medical benefits associated with these molecules [71–74]. Importantly, direct application of naringenin to cereals also promotes formation of lateral roots and improves endophytic colonisation by the diazotrophic PGPR *Azorhizobium caulinodans* [65–67]. A recent study demonstrated use of an optimised pathway for *de novo* biosynthesis of (2S)-naringenin in *E. coli* capable of producing over 100 mg/L^{-1} (2S)-naringenin from glucose [71]. While there are currently no reports pertaining to the introduction of engineered naringenin biosynthesis clusters into rhizobacteria, this simple experiment could have significant value for agricultural research.

Biocontrol

While pesticides are of significant benefit to agricultural productivity, most contain organic pollutants which persist in the environment, can be transported over vast distances, and may eventually bioaccumulate at toxic levels in organisms that occupy high trophic levels of the food-chain, including humans [75–77]. Use of plant colonising bacteria to control plant pathogens could reduce dependency on pesticides in agriculture. *Pseudomonas* spp. have been of particular interest in this regard due to their ability to competitively antagonise pathogens and produce a wide range of antifungal metabolites [78]. Four *P. fluorescens* BL915 genes *prnABCD* involved in biosynthesis of the broad-spectrum antifungal pyrrolnitrin have been expressed in the cotton colonising *P. fluorescens* strains BL914 and BL922, conveying upon them the ability to supress *Rhizoctonia solani*-induced damping-off disease [79, 80]. Additionally, the broad-spectrum antifungal 2,4-diacetylphloroglucinol (DAPG) biosynthetic gene clusters *phlDACB* from *Pseudomonas* sp. G22 and *P. protegens* Pf-

5 have been expressed in the diazotrophic wheat endophyte *Pseudomonas* sp. WS5 [81]. Culture extracts from these strains showed antagonistic effects against the fungal pathogens *Magnaporthe oryzae* B157 and *R. solani* and the strains promoted various aspects of growth for rice and sorghum infected with these pathogens.

Although often overlooked, one of the possible ramifications of engineering rhizobacteria for increased production of broad-spectrum antifungal compounds as biocontrol agents is the unintended antagonism of the arbuscular mycorrhiza (AM) symbiosis that is crucial for growth and survival of most land plants. This issue has been explored in engineered phenazine-1-carboxylic acid (PCA) producing derivatives of *P. fluorescens* SBW25 carrying the PCA biosynthesis genes *phzABCDEFG* from *P. synxantha* 2-79. The engineered PCA producing strain exhibited an improved ability to reduce *Pythium ultimum* induced damping-off disease of pea seedlings, even when the pathogen was present at over 100-fold field infestation levels [82], and promoted various growth aspects of *P. ultimum* infected pea, wheat and sugar beet [83]. Remarkably, inoculation with engineered PCA producing strain had no deleterious effect on the percentage of natural field margin pasture plants infected with AM relative to the wild-type strain SBW 25 [83]. Despite these promising early-phase results, the development of more targeted biocontrol mechanisms will be instrumental moving forward with engineering of biocontrol PGPR for agriculture. Such systems could utilise targeted release of narrow spectrum toxins and bacteriocins [84, 85] or in situ conjugal transfer of DNA sequence-specific antagonism/killing mechanisms based on CRISPR-Cas9 technology and pathogen-inducible expression systems [86–88]. The latter strategies could be particularly useful for biocontrol in the rhizosphere, which is a hotspot for horizontal gene transfer [89].

Rhizoremediation

Bacteria that sequester, detoxify, or degrade heavy metals and xenobiotic pollutants can be useful to remediate agricultural soils and reduce phytotoxicity. Engineering of rhizoremediating bacteria is a strong and rapidly expanding area of research [90–92] with several studies demonstrating direct reduction of phytotoxicity. This has been achieved for example, through heterologous expression of 1-aminocyclopropane-1-carboxylate-(ACC)-deaminases, which reduce *in planta* levels of the root elongation inhibitor ethylene that accumulates in response to various environmental stresses [93–95]. Additionally, crude transfer of the entire 108-kb *Burkholderia cepacia* G4 plasmid pTOM, encoding a toluene mono-oxygenase, into the lupin endophyte *B. cepacia* L.S.2.4, conveyed the capacity to degrade toluene in non-sterile sand and increased biomass of yellow lupine grown under toluene stress [96].

Regarding heavy metals, soil bacteria have been engineered for cell surface biosorption of Cd^{2+} using heterologously expressed eukaryotic metallothioneins (MTs) and phytochelatins (PCs). In a ground-breaking study, a mouse MT protein was fused to immunoglobulin A (IgA) protease of *Neisseria gonorrhoeae*, producing a chimeric Mtb protein which, when expressed in *Ralstonia eutropha* CH34, was transported to the cell surface [97]. The resulting strain adsorbed Cd^{2+} from the external environment with increased effectiveness and significantly decreased Cd^{2+} phytotoxicity of *Nicotiana bentamiana*. Heterologous expression of a cell-surface targeted MT in the Cd^{2+}-resistant PGPR *P. aeruginosa* Pse-w also promoted Cd^{2+} adsorption by the bacteria which, when inoculated into Cd^{2+} rich soil, increased biomass and leaf chlorophyll content of green pea [98]. In a separate study, expression of a synthetic PC in *Pseudomonas putida* 06909 improved intracellular Cd^{2+} binding and alleviated the cellular toxicity of Cd^{2+}. Inoculation of sunflower roots with this strain resulted in a marked decrease in Cd^{2+} phytotoxicity and a 40% increase in Cd^{2+}accumulation in the plant root [99].

Controlling engineered PGPR in the rhizosphere

Plant host-specific control of gene expression

During the early phases of bacterial engineering, introduced genes are commonly overexpressed at aberrantly high levels to accentuate observable phenotypes for screening under controlled laboratory conditions. Consequentially, the increased metabolic load can impair viability and fitness of the host bacteria, driving strong selection for silencing mutations. Utilisation of tuneable expression systems to regulate introduced genes is essential to preserve the native ecological characteristics of recipient bacteria and stabilise gene function [100, 101]. Utilisation of plant-derived signals to control expression of PGP genes can be particularly useful for rhizobacteria, as this strategy ensures that introduced genes are only expressed upon colonisation of the rhizosphere. Bacterial allosteric transcription factors (TF) and cognate inducible promoter pairs responding to primary and secondary metabolites present in root exudates have been identified for this purpose [45, 102]. For example, legume-derived flavonoid-inducible expression systems from rhizobia have been used to regulate PCB degradation in *P. fluorescens* [103] and N-fixation in *P. protogens* Pf-5 [45]. Signals that are prevalent in the exudates of a broader range of plants, such as salycilate, vanillate, and arabinose, have also been used to control expression of N-fixation genes in root-colonising bacteria [45, 102].

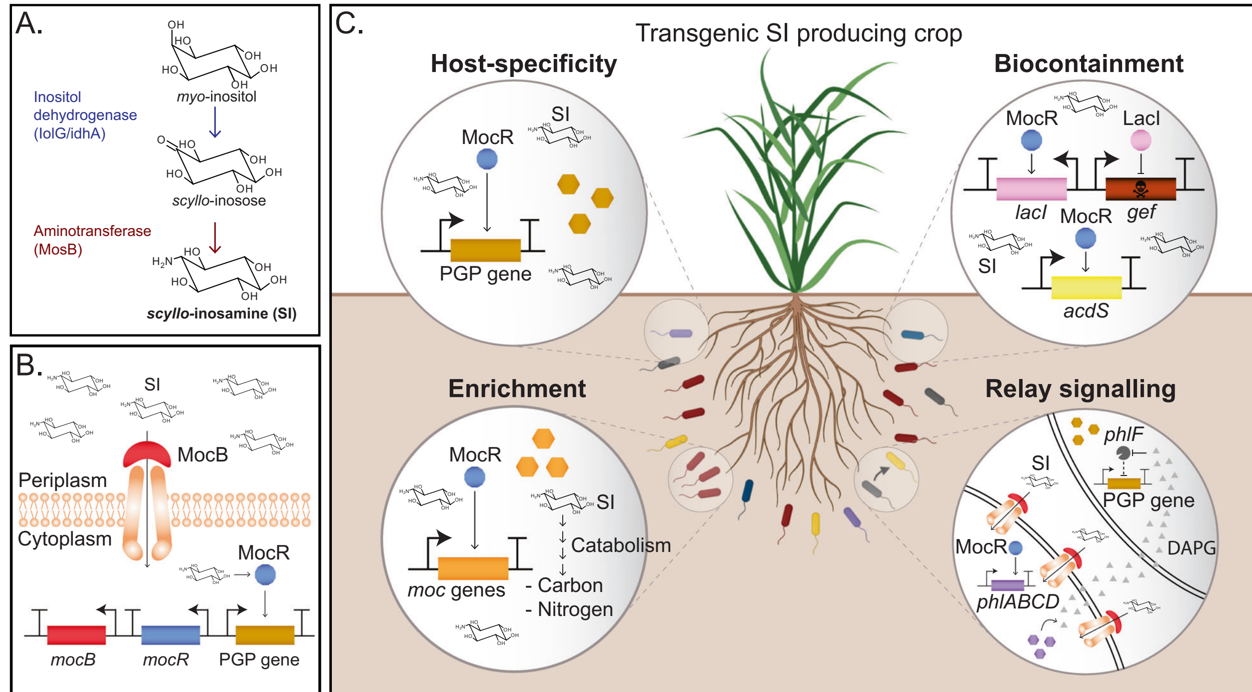

Fig. 3 Rhizopine signalling to control PGPR. a To enable signalling between plants and bacteria, a synthetic biosynthesis pathway has been engineered into barley and *Medicago* that facilitates production of the rhizopine *scyllo*-inosamine (SI) from *myo*-inositol [17]. **b** SI biosensor plasmids encoding the periplasmic rhizopine-binding protein MocB and rhizopine-dependent transcription factor MocR can be introduced into bacteria enabling genes placed downstream of the rhizopine-inducible promoter P_{mocB} to be expressed under rhizopine control. **c** SI signalling circuitry can be used to establish plant host-specific expression of rhizobacterial PGP genes, effectively coupling interactions in the field and preventing growth promotion of non-target plants. SI signalling could also be used to enrich the rhizosphere for engineered bacteria that carry the catabolic *moc* gene which permits utilisation of SI as a sole carbon and nitrogen source [108, 115, 116]. Like most signalling circuitry, the functionality of SI signalling is not ubiquitous across bacterial taxa. By bringing biosynthesis of a secondary signalling molecule such as DAPG depicted here, under SI control, the SI signal could be relayed to diverse bacteria carrying a second cognate inducible or derepressible promoter system such as the DAPG-dependent system controlled by PhlF. SI signalling could also be integrated to control multi-layered biocontainment systems such as that described by Ronchel et al. [127] where the essential *acdS* gene and *lacI* repressor, targeted for the *gef* toxin, are each expressed in response to an external stimulus. Integration of this signal could restrict proliferation of engineered rhizobacteria to the rhizosphere.

Metabolomics of plant root exudates have revealed that some plants produce unique secondary metabolites [104, 105] raising the possibility of utilising plant species-specific signals to control bacterial gene expression. This strategy offers an unprecedented opportunity to establish exclusive PGP interactions between bacteria and target plants in the field, which could prevent growth promotion of non-target weed species following promiscuous colonisation, but is currently restricted by the lack of identified cognate bacterial TFs and inducible promoters. To circumvent this restriction, regulatory mRNAs termed riboswitches can be artificially selected in bacteria to regulate gene expression in response to binding virtually any small molecule [106]. Alternatively, biosynthesis pathways for specific signalling molecules with pre-characterised cognate bacterial gene expression circuitry can be engineered into target plants and bacteria (Fig. 3a-b). Transgenic *Medicago* and barley have been developed that carry a constitutively expressed synthetic biosynthesis pathway for *scyllo*-inosamine (SI), an inositol-derived rhizopine which is naturally produced by a select few *Rhizobium* and *Sinorhizobium* strains housed within legume nodules during N-fixing endosymbiosis [17, 107, 108]. SI is well suited as plant-to-bacteria signal as it is readily exuded into the rhizosphere, but is rare in nature and both metabolically and genetically inaccessible to most bacteria [108]. Moreover, the genetic components of SI-inducible expression systems have been characterised in detail, allowing complete control of the system. SI signalling will not only be crucial for establishing control engineered PGPR traits in the rhizosphere but will be invaluable for other applications (Fig. 3c) discussed in the following subsections.

Enrichment of target bacteria

Maintaining a sufficiently large population of target bacteria in the rhizosphere remains one of the key challenges for improving plant-bacteria interactions. Pathogenic

Agrobacterium spp. have naturally evolved a successful strategy to overcome this issue, transforming plant cells to stimulate formation of crown galls or hairy roots that synthesise low molecular weight opines. Opines regulate conjugal transfer of the *Agrobacterium* pathogenic Ti plasmid and expression of pathogenicity genes [109], but are also utilised as a source of C and N by *Agrobacterium* and a narrow range of other soil bacteria [110, 111]. It has been categorically demonstrated that opine catabolising bacteria have a competitive advantage for rhizosphere colonisation of transgenic opine producing plants grown in sterile conditions [112] and are enriched in the rhizosphere of these plants grown in non-sterile soils [113, 114].

SI production by transgenic barley and *Medicago* plants may also favour proliferation of bacteria carrying natural or introduced catabolic *moc* genes (Fig. 3c) which enable utilisation of rhizopines as a sole source of C and N source [108, 115, 116]. Although this is yet to be experimentally tested, it has been demonstrated that bacteria capable of synthesising and catabolising rhizopines have a competitive advantage for nodulation of legumes [117]. Moreover, the presence of *moc* genes in natural bacteria is inherently linked with the presence of the rhizopine biosynthesis *mos* genes [108]. These findings form the basis of the "rhizopine concept" hypothesis which depicts rhizopine biosynthesis and catabolism as a form of kin selection [107]. It remains unclear as to whether introduction of a constant supply of SI in the soil would drive the rapid evolution of rhizopine catabolising bacteria, though considering that *Sinorhizobium moc* genes are encoded on the conjugative plasmid pSymA which is actively disseminated in the rhizosphere [115], this remains a distinct possibility.

Relay signalling

Like most inducible gene expression systems present in bacteria, the functionality of SI-inducible expression is not ubiquitous across taxa, restricting our ability to control gene expression in diverse PGPR. To circumvent this issue, SI or other narrow host-range signals can be relayed to incompatible bacteria through a "messenger" capable of secondary signal production (Fig. 3c). For example, acyl-homoserine lactone (AHL) biosynthesis could be placed under SI control, enabling secondary activation of quorum-sensing (QS) systems that have been designed to regulate IAA biosynthesis and N-fixation in engineered *C. pinatubonensis* and *E. coli*, respectively [45, 68]. While using specific or engineered AHL synthases and cognate receptors could avoid interference with native regulation [118], the enormous diversity of QS and quorum-quenching bacteria present in the rhizosphere would undoubtedly result in significant interference of QS circuits [119]. The same problem rings true for other engineered extracellular signalling molecules such as DAPG, DHBA and naringenin [71–74, 81, 100, 120], which have been used to control N-fixation in diverse bacteria [45]. This problem highlights a pressing need for the development of heterologous and/or synthetic bacteria-to-bacteria signalling circuitry that can be used in the rhizosphere to control gene expression.

Biocontainment

Escape of engineered PGPR and their genetic material from the intended environment has potential to endanger natural community dynamics. To prevent this escape, biocontainment mechanisms can be embedded within rhizobacterial genomes that restrict viability and proliferation outside of the target rhizopshere and block horizontal gene transfer (HGT) [121–123]. One strategy involves implementing synthetic auxotrophy through deletion of one or more essential metabolic genes, such that the bacteria becomes dependent on an exogenous supply of a metabolite for growth and survival. For biocontainment in the rhizosphere, this strategy would require the target plant to exude essential metabolites at high enough concentrations to support growth of the auxotrophic bacteria. Alternatively, plant-to-bacteria signals such as SI could be used to activate expression of essential genes [124, 125] or control expression of toxins or genetic "kill-switches" such as deadman and passcode [126, 127] (Fig. 3c). While such single biocontainment mechanisms rarely satisfy the U.S. National Institute of Health's recommended safety criteria of an escape frequency below 1 in 10^8 cells [128, 129], combining strategies can be highly effective. An escape frequency of <1 in 10^9 cells was achieved for *P. putida* MC8, which encoded an essential 3-methylbenzoate inducible aspartate-semialdehyde dehydrogenase gene (*asd*) gene and repressible *gef* toxin [127]. MC8 colonised the rhizosphere of maize seedlings doused with 3-methylbenzoate at levels reminiscent of the wild-type strain and was not detectable after 25 days in the absence of the pollutant. An escape frequency of <1 in 10^{12} cells has also been achieved using chemically-inducible riboregulators to control expression of the essential gene *glmS* in combination with an engineered addiction module capable of cleaving the *E. coli* chromosome [125].

Prospects for utilisation of engineered rhizobacteria in agriculture

As we have alluded to throughout this review, the use of genetically modified (GM) organisms (GMOs) could be instrumental for the sustainable intensification of agriculture. However, there remains significant public concern surrounding their use, and at present, release of GMOs in

most countries is strictly regulated [130]. GM crops have been commercially produced for over two decades, being planted over 191.7 million hectares by 17 million farmers in 26 countries in 2018 [131]. Despite this, a recent report by the U.S. National Academies of Sciences, Engineering, and Medicine found little evidence to connect GM crops with adverse agronomic or environmental problems compared to current agricultural practices, and found no evidence implicating a higher risk to human health from eating GM foods compared to non-GM counterparts [132]. Compared to plants, bacteria can be more elaborately engineered, are faster growing, and are more prone to horizontal gene transfer, making them potentially more difficult to manage and monitor. Nevertheless, since the first field trials in the Netherlands, 1986, comparing the effects of siderophore producing *P. putida* strains against genetically modified siderophore null Tn*5* mutants [133], the release of GM bacteria has been studied for over three decades without notable environmental impacts, suggesting that they might be safely used [128, 134].

Today, many scientists argue that current regulations for risk assessment of GMOs fail to use a scientifically defensible approach and do not account for the level of actual hazard or risk [128, 135, 136]. Because foreign inoculant bacteria in agricultural settings are typically outcompeted by the natural microbiota, it seems plausible that risk assessment of parental strains may be sufficient to evaluate the prospects and impacts of some GM bacteria [128]. With continued development of molecular tools such as CRISPR for "markerless" genome editing [137], combined with novel strategies to establish stringent biocontainment of bacteria [121] and their genetic material [138–140], the prospect of creating safe GM bacteria that can be controlled in the environment has never been closer. Considering our pressing need to transition into more sustainable agricultural practices, while at the same time increasing food production, now is the ideal time for a thorough review of global regulations governing release of GMOs in agriculture.

Concluding remarks

Over the last few decades, the development of novel genetic engineering and synthetic biology tools have driven unprecedented advances regarding engineering and transfer of PGPR traits. With a suite of successfully engineered PGP traits and vectors for transfer available, the research focus is beginning to shift towards optimisation of engineered PGPR to develop strains which do not suffer the ecological shortfalls of their natural progenitors (Fig. 1). Many laboratories now routinely test constructs in resilient and competitive rhizobacteria isolated from the environment, and attempt to more accurately replicate field conditions in their experimental design. Tuneable expression systems are now frequently implemented into genetic circuitry permitting more robust control over engineered traits and reducing metabolic load on the host. With the advent of synthetic host-specific plant-to-bacteria SI signalling [17], it has become possible to exclusively couple engineered bacteria-plant interactions in the field, which could prevent the possibility for growth promotion of non-target plant species following promiscuous colonisation. Expanding the functionality of SI signalling in the rhizosphere through use of secondary signals, and utilising SI signalling to both enrich the rhizosphere for target bacteria and establish stringent biocontainment will represent key milestones in the development of more effective and safe engineered PGPR. Through integration of the technologies discussed in this review, we envisage the future possibility of constructing entire synthetic communities of engineered PGPR as biostimulants for crops grown under given conditions. Ensuing a global overhaul of restrictions governing the release of GMOs in the environment, utilisation of tailored synthetic communities could play a crucial role in the sustainable intensification of agriculture over the coming years.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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