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Microbiome engineering for sustainable agriculture: using synthetic biology to enhance nitrogen metabolism in plant-associated microbes

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Plants benefit from symbiotic relationships with their microbiomes. Modifying these microbiomes to further promote plant growth and improve stress tolerance in crops is a promising strategy. However, such efforts have had limited success, perhaps because the original microbiomes quickly re-establish. Since the complex biological networks involved are little understood, progress through conventional means is time-consuming. Synthetic biology, with its practical successes in multiple industries, could speed up this research considerably. Some fascinating candidates for production by synthetic microbiomes are organic nitrogen metabolites and related pyridoxal-5′-phosphate-dependent enzymes, which have pivotal roles in microbe–microbe and plant–microbe interactions. This review summarizes recent studies of these metabolites and enzymes and discusses prospective synthetic biology platforms for sustainable agriculture.

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Introduction

Symbiotic plant–microbe interactions benefit both participants. While plants provide nutrition, their associated microbes provide essential metabolites for plant growth and stress tolerance. However, these metabolisms are not yet well understood. Elucidating microbial metabolisms and their regulation is therefore critical for realizing new opportunities in sustainable agriculture.

Plant–microbe interactions help plants in many ways. For example, diazotrophic microbes convert dinitrogen gas (N_2) into plant-available nitrogen (N) (e.g. NO_3^- and NH_4^+), which promotes plant growth (biofertilization). Some microbes produce plant hormones and/or metabolites similar to plant hormones that support plant growth and development (biostimulation). Additionally, microbe-produced volatile organic compounds (VOCs) [1] can improve plants' ability to tolerate both biotic and abiotic stressors, including insects, pathogens, drought, and extreme temperature.

Microbe–microbe interactions also play vital, although indirect, roles in plant–microbe interactions. Microbial metabolites mediate microbe–microbe interactions through metabolic exchanges and complementation, as well as by functioning as chemical signals. For example, quorum-sensing molecules (e.g. *N*-acyl homoserine lactones) promote formation of biofilms that enhance microbe survival, production of other specialized metabolites, cell motility, and root development [2]. Antimicrobials control growth of microbes that may be pathogenic to plants (biocontrol).

Overall, symbiotic relationships among microbes and plants form the core of biomes. Modulating these relationships is emerging as a potential strategy for making agriculture more sustainable [3].

Many research efforts have focused on discovering plant-growth-promoting rhizomicrobes (PGPRs). Application of PGPRs to agricultural fields, however, has yielded inconsistent benefits for various crop species and cultivation conditions, perhaps because the pre-existing microbiomes are resilient and soon overgrow the introduced species [4]. This limitation is extremely hard to overcome because of the complex interactions involved.

Synthetic biology has emerged as a promising strategy because it facilitates targeted engineering of key microbial strains, thereby harnessing the microbiome's

Figure 1
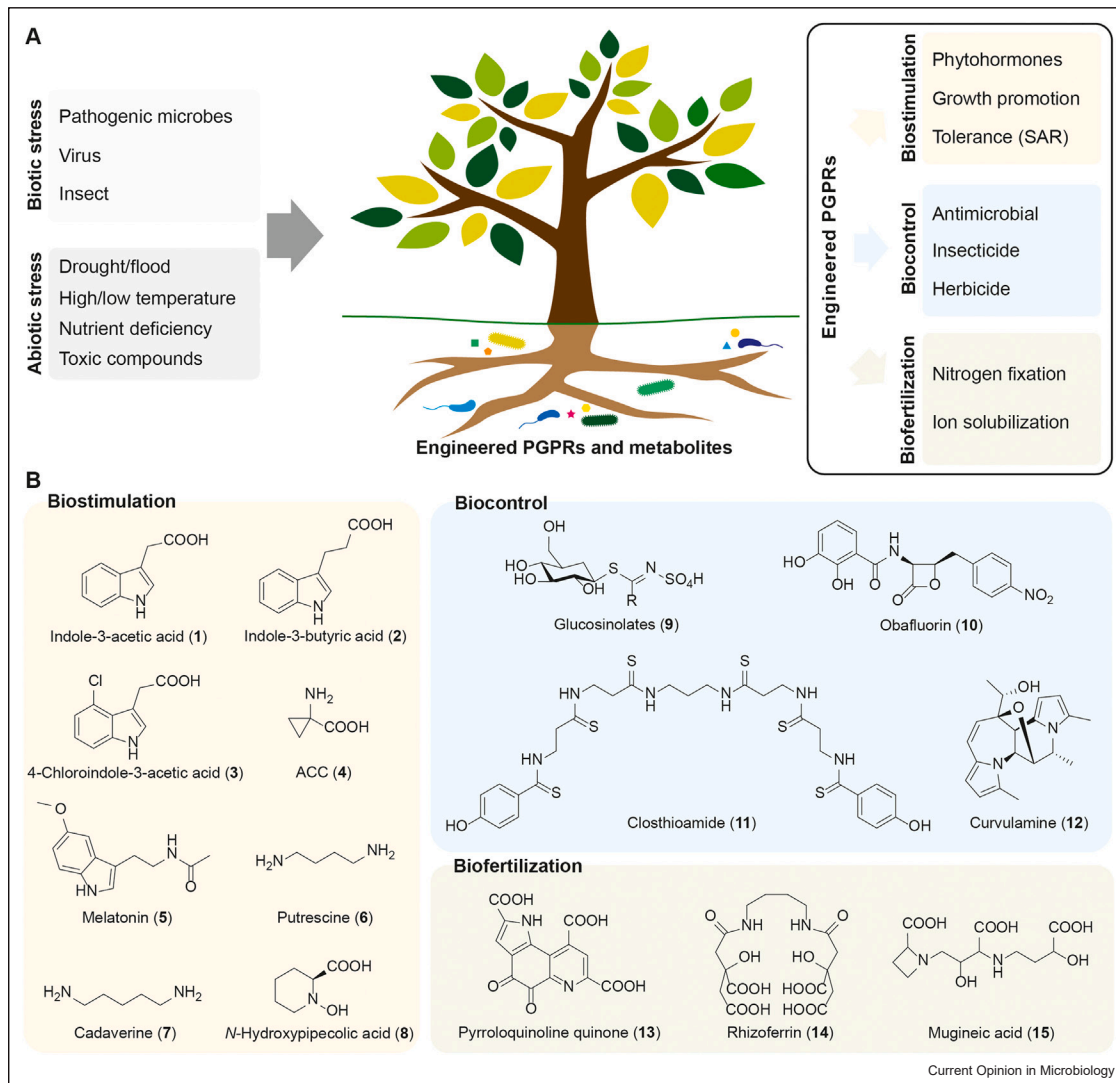
Engineering of PGPRs for sustainable agriculture: **(a)** PGPRs as biological sources of phytohormones (biostimulation), pesticides (biocontrol), and nutrients (biofertilization) under biotic and abiotic stresses and **(b)** functional N metabolites discussed in this review.

power to increase sustainability in agriculture. Both new strains that can robustly colonize diverse plant species and strains from original communities may be engineered to improve plant growth without chemical fertilizers and pesticides (Figure 1a). Introducing plant-growth-promoting (PGP) traits to these strains may provide consistent benefits, regardless of changes in crop species or cultivation conditions [5–7].

Researchers often attempt to understand metabolic networks, as well as microbe–microbe and plant–microbe interactions and their regulation, by investigating carbon (C) metabolism. However, N metabolism has been explored less, despite the versatile roles of N metabolites in cellular signaling and physiology. This

review therefore focuses on the roles of organic N metabolites derived from microbes, as well as on the roles of pyridoxal-5′-phosphate (PLP)-dependent enzymes, which constitute the primary family of enzymes responsible for N metabolism. Additionally, this review highlights potential applications of N metabolites and related gene products developed using synthetic biology to engineer new PGPRs that could improve the sustainability of agriculture.

Nitrogen-containing metabolites

As building blocks of proteins and precursors for diverse metabolites, amino acids are essential N metabolites in all living organisms. Aside from 22 amino acids used to build proteins, a wide variety of nonproteinogenic amino

acids are essential in cellular metabolism and physiological maintenance. For example, β-alanine and 5-aminolevulinic acid are building blocks of Coenzyme A and chlorophyll [8], respectively, and 1-aminocyclopropane-1-carboxylic acid (ACC) (4) is a precursor of ethylene, a phytohormone associated with plant senescence (e.g. fruit ripening) and stress response [9] (Figure 1b). Also, L-tryptophan and its analogs are precursors of endogenous auxins (e.g. indole-3-acetic acid (1), indole-3-butyric acid (2), and 4-chloroindole-3-acetic acid (3)) [10]. These auxins are essential for plant growth and development.

Ornithine, citrulline, and γ-aminobutyric acid are precursors for various metabolic networks and are converted to bioactive-specialized metabolites (e.g. alkaloids) [11]. Canavanine and *m*-tyrosine are important for alleviating cellular oxidative stress [12,13], and betaines, derived from glycine or β-alanine, confer salt tolerance in plants [14]. Also, nicotianamine is a precursor of phytosiderophores, mugineic acid (15), and its derivatives, for iron chelation [15]. Recently, *N*-hydroxypipecolic acid (8) was identified as a critical inducer of the systemic acquired resistance immune response in plants [16]. Proteinogenic L-amino acids can be isomerized to D-amino acids important for biofilm formation. For example, D-alanine and D-glutamate are key molecules in peptidoglycan layers in Gram-negative bacteria [17]. D-Amino acids are also important for synthesis of specialized metabolites (e.g. nonribosomal peptides) [18]. These metabolites may function in communication between biome members and may help microbial producers survive in competitive resource-scarce environments.

Decarboxylation of amino acids or amination of carbonyl compounds (i.e. aldehydes and ketones) generates biogenic amines, which are important in cell signaling. For example, melatonin (5) acts as a hormone in plants and fungi [19–21]. Although their biological functions are poorly understood, polyamines have been identified as phytohormones coordinating cellular activities and mediating plant–pathogen interaction in plants. For example, researchers found that cadaverine (7), a polyamine, inhibited root growth by inducing biotin limitation [22].

Microbes and plants often produce complex organic N compounds (e.g. alkaloids) exhibiting diverse physiological activities. Notably, using alkaloids for their antimicrobial and antiviral effects has been proposed as a sustainable approach to protecting plant health [23,24]. Some organic N compounds can confer substantial biological activity, even in small quantities. For example, most vitamin-B derivatives serve as organic cofactors (e.g. PLP, NAD^+, and FAD^+), and their deficiency induces metabolic disorders. In addition, a pyrroloquinoline quinone (PQQ) (13), a cofactor synthesized by some

PGPRs, affects PGP traits by modulating redox state as well as through its role in synthesis of sugar acids useful for solubilization of inorganic phosphorus [25–27].

Moreover, N-containing pigments (e.g. chlorophyll) are essential for photosynthesis, allowing plants to absorb energy from light. In addition, some plants have unique specialized metabolites (e.g. glucosinolates) bearing N and sulfur. Glucosinolates (9), which are derived from various amino acids, are essential to plant immune systems and can function as natural pesticides [28]. In the next section, we review the biological pathways and enzymes involved in synthesizing important organic N metabolites.

Enzymes in nitrogen metabolisms

Although modern agriculture relies heavily on chemical fertilizers as an N source, plants can also source bioavailable N (e.g. NO_3^-, NH_4^+, and organic N compounds) through ecological relationships with microbes that can directly fix atmospheric nitrogen (N_2) [29]. N catabolism in microbes proceeds in four phases: (1) fixation, (2) assimilation, (3) distribution, and (4) biotransformation. Plants can take up N metabolites yielded at any phase, and may proceed with the subsequent phases, utilizing the N metabolites for their development and growth.

N-fixation converts N_2 into ammonia (NH_4^+), which is accessible to most living organisms. Major N-fixation enzymes include dinitrogenase and its reductase, which exploit molybdenum–iron, vanadium–iron, or iron–iron cofactors [30]. The fixed NH_4^+ molecules enter into the N-assimilation phase for synthesis of organic N compounds, but can also undergo nitrification, anammox, and/or denitrification in the microbial nitrogen cycle, and be converted into diverse inorganic forms (Figure 2) [30]. In the N-assimilation phase, NH_4^+ is incorporated into organic N compounds such as glutamine and glutamate, catalyzed by NAD(P)H-dependent glutamate dehydrogenase and ATP-dependent glutamine synthetase [31]. In the case of nitrate, metal-dependent nitrate and nitrite reductases are additionally employed to prepare NH_4^+ [30].

In the distribution and biotransformation phases, in which assimilated N is converted into primary and specialized metabolites, glutamate synthase (also known as glutamine:2-oxoglutarate aminotransferase) and PLP-dependent aminotransferases (ATs, enzyme commission [EC] 2.6.1) distribute N throughout the metabolic networks [32,33]. PLP can form a Schiff-base linkage with the amino group. This unique property makes PLP a versatile cofactor for enzymes associated with N metabolism. In fact, PLP-dependent enzymes distribute across five of the seven EC classes: EC1 (oxidoreductases), EC2 (transferases), EC3 (hydrolases), EC4

Figure 2
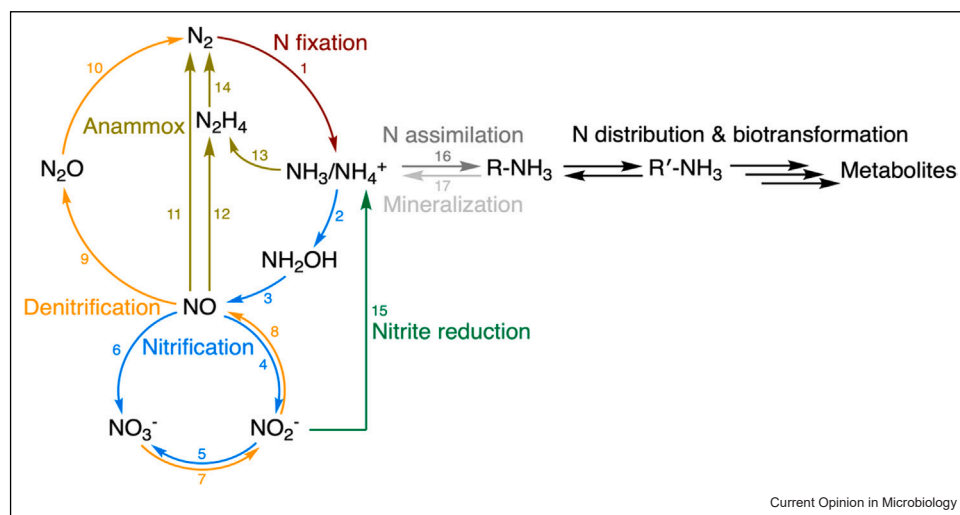
N metabolism in microbes: **(a)** N fixation (step 1, brown), **(b)** nitrification (steps 2–6, blue), **(c)** denitrification (steps 7–10, orange), **(d)** anammox (steps 11–14, gold), **(e)** nitrite reduction (step 15, green), **(f)** N assimilation (step 16, dark gray), **(g)** mineralization (step 17, light gray), **(h)** N distribution and biotransformation (black).

(lyases), and EC5 (isomerases) (Figure 3). Additionally, 1.6% of all reported enzymes in UniProtKB (October 2021) are annotated as PLP-dependent. Most enzymes involved in ATs reactions employ PLP as a cofactor, and 73% of all enzymes predicted to catalyze transaminase reactions have PLP-binding motifs. These observations strongly suggest the core functions of PLP-dependent enzymes in N metabolic networks.

N metabolism after the assimilation phase is poorly understood because of the networks' complexity. In particular, assigning each reaction is difficult because ATs have broader substrate specificity than do most other enzymes, and because function overlaps significantly among different ATs. These metabolic redundancies may be important to create robust N networks for production of essential metabolites whose demands could proportionally shift, depending on plants' growth and their developmental phases, as well as on their physiological responses to environmental changes. Besides increasing understanding of N-network regulation, designing microbial systems that can help plants shift their optimal N metabolic networks could promote plant growth [6,7].

Molecules that confer PGP traits may be classified as (1) phytohormones (biostimulation), (2) nutrients (biofertilization), or (3) antimicrobials (biocontrol). Native symbiotic communities produce these molecules, but in addition, recent studies have shown promising results from microbiome engineering in modulating bioactive N metabolites in order to promote plant growth [24,34–38]. In the following section, we discuss potential

applications of PLP-dependent enzymes for improving agricultural sustainability via microbiome engineering.

Potential applications of pyridoxal-5′-phosphate-dependent enzymes in biostimulation

Phytohormones are among the well-known metabolites involved in plant growth and development. They influence plant physiology in many ways. To date, several microbes engineered to produce phytohormones have shown promising ability to promote plant growth. For example, Zúñiga et al. engineered *Cupriavidus pinatubonensis* to produce the auxin indole acetic acid (IAA) (**1**) under the control of a quorum-sensing signal, enhancing the root growth of *Arabidopsis thaliana* [34]. In addition to IAA (**1**), plants synthesize endogenous auxins (e.g. indole-3-butyric acid (**2**) and 4-chloroindole-3-acetic acid (**3**)) [10], which may also be useful if provided by microbes. Recently, the β-subunit of tryptophan synthase (TrpB, EC 4.2.1.20), which is involved in biosynthesis of tryptophan via a β-replacement reaction between L-serine and indole, has emerged as useful for synthesis of aromatic amino acids via artificial evolution. For example, Rix et al. constructed various TrpB variants for tryptophan derivatives using an *in vivo* high-throughput evolution technique with orthogonal DNA polymerase [39]. Broad substrate specificies of TrpB variants potentially enable production of diverse unnatural auxins.

Ethylene, a multifunctional phytohormone involved in plant growth and senescence [9], can be modulated by ACC deaminase (EC 3.5.99.7) via ACC (**4**) degradation. This reaction can be used to influence growth and other

Figure 3

Current Opinion in Microbiology
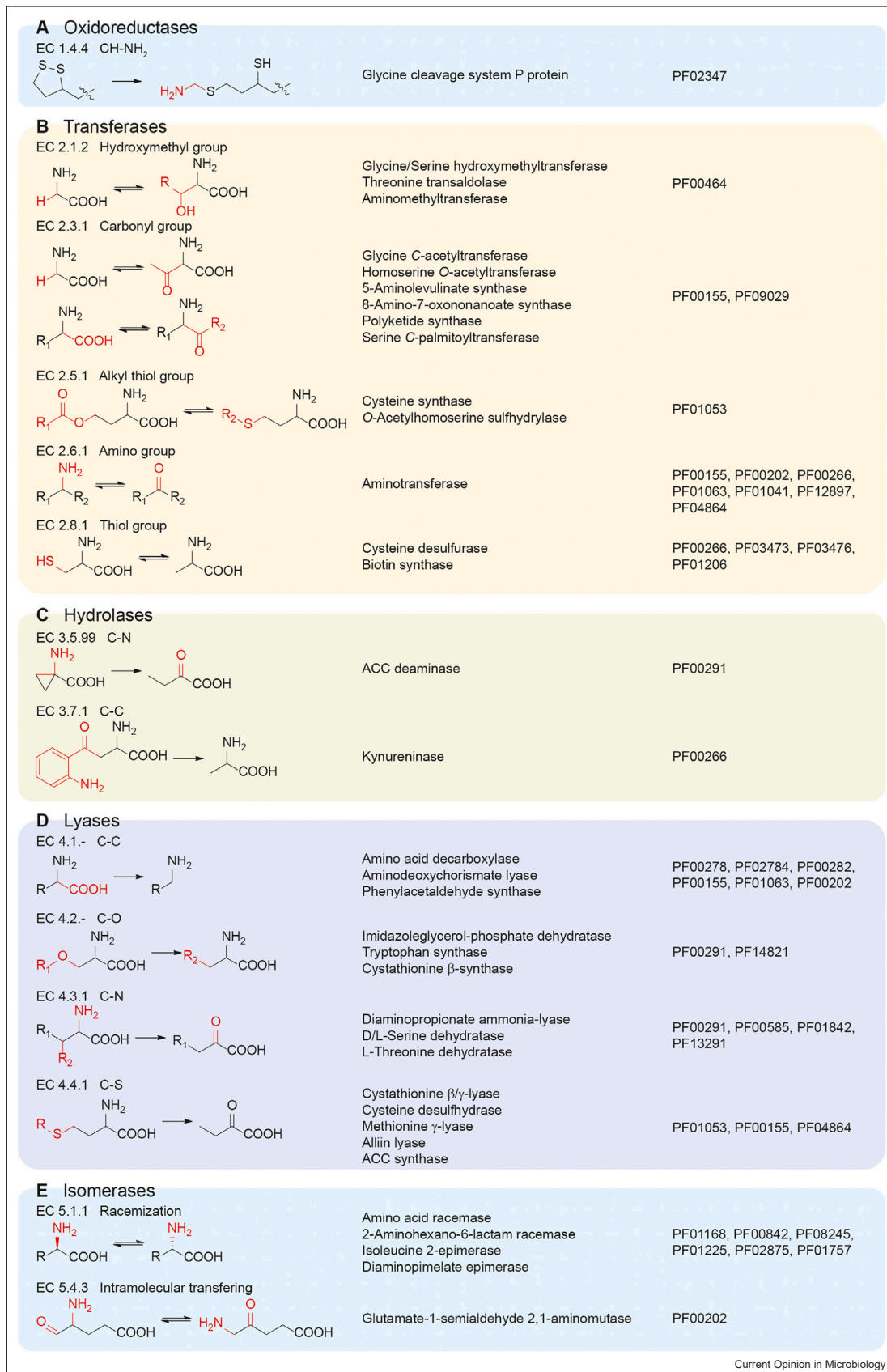
Biochemical features and structural clusters of PLP-dependent enzymes. Each column represents biochemical reactions (left, EC1–5), representative enzymes (middle), and relevant Pfam structural domains (right). The chemical moieties in red indicate where catalytic reactions occur.

processes in plants. In one experiment, chickpeas were inoculated with microbes that heterologously expressed ACC deaminase [35], resulting in growth promotion of chickpea. More recently, endophytic microbes were engineered to display ACC deaminase, which conferred saline and wilt resistances to rice and banana plants, respectively, and also promoted their growth [36,37].

Chen et al. elucidated the function of L-cysteine desulfhydrase (EC 4.4.1.28) in abscisic acid signaling, which is critical in plant response to drought stress. Hydrogen sulfide (H_2S) generated by L-cysteine desulfhydrase regulates plant signaling pathways through post-translational modification [40••]. Ser/Cys synthase (EC 2.5.1.47), which catalyzes reversible β-replacement between L-serine and L-cysteine, may be an alternative for synthetic control of cellular H_2S [41]. As H_2S can lead to both positive and negative effects, depending on its cellular concentration [42], its precise control based on serine/cysteine metabolism may be a promising strategy for sustainable agriculture.

Biogenic amines serve as hormones in plants [21,22]. Bacteria can be engineered to produce amino acid decarboxylase (AADC) to produce these amines. For example, putrescine (**6**) and cadaverine (**7**) are generated by a single decarboxylation step from ornithine and lysine, respectively. Melatonin (**5**) is synthesized from 5-hydroxy-L-tryptophan by AADC (EC 4.1.1) coupled with serotonin-*N*-acetyltransferase and acetyl-serotonin *O*-methyltransferase [21]. Bacterial AADCs with broad substrate specificities have been used to produce diverse monoamines that are precursors of biocontrol and biostimulation agents such as alkaloids and catecholamines [43,44]. Aromatic aldehyde synthases (EC 4.1.1.107–109), specific clades of AADCs especially lacking a catalytic Tyr residue [45], can catalyze oxidative deamination of aromatic amino acids to yield VOCs such as phenylacetaldehyde, a nematocidal agent and mediator of plant–insect interaction [46]. These various functions reveal the broad potential of AADCs to provide microbes with biostimulation and biocontrol activities.

In the next section, we review potential uses of PLP-dependent enzymes for biocontrol and biofertilization.

Potential applications of pyridoxal-5′-phosphate-dependent enzymes in biocontrol and biofertilization

Microbes engineered to produce siderophores may help source essential recalcitrant metal ions from fields for use by plants. For example, rhizoferrin (**14**) is a siderophore produced in fungi and bacteria. Li et al. revealed the biosynthetic route for rhizoferrin (**14**) from ornithine and citrate by a nonribosomal peptide synthetase-independent siderophore synthetase and PLP-dependent *N*-citrylornithine decarboxylase [47•]. Plants in the *Poaceae* family were previously engineered to express PLP-dependent nicotianamine ATs to make phytosiderophores, mugineic acid (**15**), and its derivatives, resulting in significantly improved ability to acquire metal ions [15]. Pathways for rhizoferrin (**14**), phytosiderophores (**15**), and other siderophores could also be introduced into microbes such as PGPRs to increase the availability of metal ions. Moreover, combining siderophore-mediated and PQQ-mediated routes in PGPRs could be an attractive approach to enhancing plant mineral uptake [26,27].

Microbes engineered to produce antibiotics have also proved their ability to protect plants from biotic stresses [24,38]. Recently, Schaffer et al. and Scott et al. found that L-threonine transaldolase (LTTA) catalyzed formation of β-hydroxy-α-amino acids and was essential for biosynthesis of (2*S*,3*R*)-2-amino-3-hydroxy-4-(4-nitrophenyl)butanoate in the plant-associated bacterium *Pseudomonas fluorescens*. This metabolite has biocontrol properties and is also a precursor for the antibacterial agent obafluorin (**10**) [48]. Xu et al. engineered *Pseudomonas* sp. to produce LTTA that could catalyze formation of (2*S*,3*R*)-β-hydroxy-α-amino acids with the phenyl group at the β-position substituted with phenyl-group derivatives. These unnatural specialized metabolites may be used as precursors for diverse antibacterial agents [49]. However, the low stereoselectivity of the engineered LTTA at the β-position limits the utility of this bioconversion owing to the unknown effect of side products. Nevertheless, this work represents progress toward engineering microbes as novel biocontrol agents.

Dai et al. discovered a novel fungal BGC for synthesis of antibacterial alkaloids (e.g. bipolamine and curvulamine (**12**)). In this BGC, CuaB exploits PLP as a cofactor to catalyze formation of C−C and C−O bonds via Claisen condensation and oxidation, respectively [50•]. This study also determined that ATs CtaB participated in the biosynthesis of an antibiotic peptide, closthioamide (**11**), by amination of the aldehyde group [51]. These BGCs may be introduced into symbiotic microbes as biocontrol agents.

As discussed, PLP-dependent enzymes participate in production of a wide variety of metabolites. However, an excessive supply of specific metabolites may burden plant growth. Therefore, engineering of microbes to

produce the metabolites discussed in this section must be carefully balanced. The following section discusses potential synthetic platforms for precisely modulating primary and bioactive-specialized metabolites that could contribute to developing more-sustainable agriculture.

Future perspective of synthetic platforms for sustainable agriculture

Synthetic biology has been used to directly engineer key members of microbiomes that provide plants with consistent benefits [5–7]. Because symbiosis-based plant breeding and use of molecular biology techniques to engineer microbiomes have been reviewed extensively elsewhere [6,7,52], this section focuses on potential microbial hosts and regulatory circuits for practical applications of PLP-dependent enzymes and related BGCs for sustainable agriculture.

As key members of plant-associated microbiomes, methanotrophs (methane-utilizing bacteria) have recently gained attention as synthetic platform hosts. Because they natively possess the ability to fix both N_2 and CO_2 [53], they are attractive targets for adding further PGP traits and establishing as synthetic PGP microbes. Liu et al. recently engineered methanotrophs that could biodegrade herbicide contaminants [54••], and Pham et al. engineered them to produce IAA (**1**); inoculation of wheat with these engineered methanotrophs accelerated plant development [55••]. While practical applications need to be developed, use of methane to control cell proliferation in methanotrophs may be a viable strategy for addressing biosafety concerns [54••]. Wang et al. recently reported an O_2-insensitive bacterial methane synthesis in which a PLP-dependent AT converted methylamine into methane in the presence of α-ketoglutarate [56•]. Although the detailed reaction mechanism has not yet been identified, discovery of this distinct function of a methane-producing PLP-dependent enzyme could presage a breakthrough in the practical application of methanotrophs.

Diversifying genetic regulatory modules can allow delicate control of synthetic pathways that are activated on demand according to host plant physiology. Fascinating potential targets for dynamic regulation are small molecules involved in plant–microbe interactions and plant stress response. Ryu et al. recently constructed biosensors for natural and non-natural signaling molecules (e.g. sugar, phytohormones, quorum-sensing molecules, and orthogonal chemical signals) that enabled control of N fixation in various microbes [5••].

More recently, Herud-Sikimić et al. engineered an *E. coli* Trp repressor to a FRET-based auxin biosensor that undergoes conformational change in the presence of auxin-related molecules but not L-tryptophan [57•]. Because the conformational change induced by L-tryptophan is a core function in the Trp operon [58], the engineered Trp repressor may allow auxin-dependent biosynthesis. Developing dynamic regulatory circuits for controlling expression of PGP traits may help maintain the viability of engineered host microbes in pre-existing microbiomes and thereby facilitate their potential contributions to sustainable agriculture.

In nature, plants interact with multiple PGPRs whose properties may work cooperatively to provide benefits. For example, Kumar et al. observed synergistic effects of ACC deaminase- and siderophore-producing PGPRs that enhanced sunflower growth [59••]. This result implies that layering PGP traits in a host strain under single or multiple regulatory circuits may maximize their advantages. Furthermore, microbiome engineering inspired by native PGPR colonization, for example,

Table 1

Applications of microbiome engineering to sustainable agriculture described in this review.

Target molecules	Host microbes	Notes	Ref.
Biostimulation			
Indole-3-acetic acid	*Cupriavidus pinatubonensis* JMP134	Quorum-sensing signal-dependent IAA production improved *Arabidopsis* root growth	[34]
Indole-3-acetic acid	*Methylotuvimicrobium alcaliphilum* 20Z	Engineering for L-tryptophan and IAA promoted wheat development under saline–alkaline conditions	[55••]
Ethylene	*Mesorhizobium ciceri*	Introduction of *Pseudomonas putida* ACC deaminase gene increased chickpea plant growth	[35]
Ethylene	*Enterobacter* sp. E5, *Kosakonia* sp. S1	Surface expression of *Pseudomonas fluorescens* ACC deaminase increased salt tolerance of rice sprout	[36]
Ethylene	*Enterobacter* sp. E5, *Kosakonia* sp. S1	Surface expression of *Pseudomonas fluorescens* ACC deaminase improved *Fusarium* wilt resistance of banana	[37]
Biocontrol			
2,4-Diacetylphloroglucinol	*Pseudomonas* sp. WS5	Introduction of *Pseudomonas protegens* BGC for 2,4-diacetylphloroglucinol enabled rice, sorghum, and wheat to grow under biotic stress	[38]
Mycosubtilin	*Bacillus subtilis* BBG100	Constitutive expression of mycosubtilin-producing BGC conferred *Pythium* pathogen resistance to tomato	[24]

Table 2

Summary of recent studies of PLP-dependent enzymes with potential for agricultural applications.

Enzyme	Target molecules	Notes	Ref.
Tryptophan synthase β-subunit	L-Tryptophan derivatives	Expanding substrate specificity via continuous directed evolution	[39•]
Ser/Cys synthase	H_2S	Reversible conversion between Cys/H_2O and Ser/H_2S	[41]
AADC	Aliphatic monoamines	Broad substrate specificity toward aliphatic amino acids	[43•]
	Aromatic monoamines	Broad substrate specificity toward aromatic amino acids	[44]
	Aromatic monoamines, benzylisoquinoline alkaloids	Altering catalytic performance (e.g. substrate specificity, catalytic reaction)	[45•]
	Rhizoferrin	Cascade reaction with nonribosomal peptide synthetase-independent siderophore synthetase	[47•]
LTTA	Obafluorin	BGCs for obafluorin in *P. fluorescens* ATCC 39502	[48]
	(2S, 3R)-β-Hydroxy-α-amino acids	Activity enhancement for diverse aromatic aldehydes via directed evolution	[49]
CuaB	Curvulamine, bipolamine	BGCs for curvulamine	[50••]
CtaB	Closthioamide	BGCs for closthioamide	[51]

through siderophore-utilizing ability [60••,61], may open a new era for sustainable agriculture via customized PGPR consortia.

Conclusions
Symbiotic interactions between plants and microbes have been exploited to make agriculture more sustainable. However, this approach has not yet benefited plant growth consistently, so wider application has been hampered. Synthetic biology, having introduced breakthroughs in efficient engineering of microbes to produce diverse bioproducts, can be used in similar ways to create microbes to advance sustainable agriculture. The central roles of N metabolites in plant physiology make N metabolic enzymes compelling targets for engineering. Although recent studies suggest the feasibility of using engineered microbes to improve crops, more research is needed to further elaborate synthetic biology approaches to creating more-reliable PGP strains that support more-sustainable agriculture (Tables 1 and 2).

Conflict of interest statement
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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- of special interest
- of outstanding interest.

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