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Review

Microbiome Engineering: Synthetic Biology of Plant-Associated Microbiomes in Sustainable Agriculture

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To support an ever-increasing population, modern agriculture faces numerous challenges that pose major threats to global food and energy security. Plant-associated microbes, with their many plant growth-promoting (PGP) traits, have enormous potential in helping to solve these challenges. However, the results of their use in agriculture have been variable, probably because of poor colonization. Phytomicrobiome engineering is an emerging field of synthetic biology that may offer ways to alleviate this limitation. This review highlights recent advances in both bottom-up and top-down approaches to engineering non-model bacteria and microbiomes to promote beneficial plant–microbe interactions, as well as advances in strategies to evaluate these interactions. Biosafety, biosecurity, and biocontainment strategies to address the environmental concerns associated with field use of synthetic microbes are also discussed.

Highlights

Mutualistic microbes associated with plants have enormous potential for economical and sustainable agriculture.

There are two approaches to plant microbiome engineering: the bottom-up approach that involves isolating, engineering, and reintroducing specific microbes, and the top-down approach that involves synthetic ecology, using horizontal gene transfer to a broad range of hosts *in situ* and then phenotyping the microbiome.

Recent advances in genome engineering tools, meta-omic tools, computational tools, and genome-wide functional genomics can improve our ability to engineer microbes for biocontrol, biofertilization, and biostimulation, as well as enhanced crop productivity and yield.

Various devices can facilitate the evaluation of genetically modified microbes before field studies.

Robust biosafety, biosecurity, and biocontainment strategies need to be developed for use of genetically modified microbes in the environment.

Phytomicrobiome Engineering for Sustainable Agriculture

The United Nations estimates world population will be 9.8 billion people by 2050 (https://population.un.org/wpp/). Agricultural productivity must increase by an estimated 70% to meet increasing demand for food, feed, fiber, and bioenergy (Global Agricultural Productivity Initiative: https://globalagriculturalproductivity.org/). Because arable acreage is unlikely to grow [1], meeting this demand requires achieving higher yields, currently attempted using artificial fertilizers and pesticides whose manufacture and use are not sustainable. Synthetic nitrogen (N) fertilizer production is energy-intensive [2]. Phosphorous (P) and potassium (K) fertilizers are mainly produced from finite mined resources likely to be depleted within 100 years. Pesticides with carcinogenic, developmental, and environmental risks are restricted [3,4]. More sustainable strategies to achieve ever-higher crop yield are urgently needed.

Plant-associated microbes harbor enormous potential to provide economical and sustainable solutions to current agricultural challenges. Although plants provide diverse ecological niches for microbes [5,6], microbes provide **plant growth-promoting (PGP) traits** (see Glossary) for plants [7]. Many PGP microbes have been isolated, and some are widely accepted as biofertilizers, biostimulants, and biocontrol agents (www.cropscience.bayer.com/innovations/agriculture-biologicals/a/hidden-helpers-below-ground). However, applying PGP microbes to fields for commercial adoption has had limited success [8–12]. This is likely because the new microbes are excluded by the more-resilient existing microbial communities [13], whose composition has been shaped over time through complex multilateral interactions with the environment [14–20]. Finding new microorganisms that can sustainably support plant development, nutrition, fitness, disease control, and productivity in dynamic and stressful environments therefore depends on developing strategies to manage **phytomicrobiomes** [5,15,21–24].

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Meta-omic studies and computational tools offer researchers many ways to increase our understanding of interactions among phytomicrobiomes. Knowledge derived from these studies may provide strategies for using PGP microbes in fields [25]. However, such strategies will likely only be effective under limited conditions because phytomicrobiomes are extremely complex, heterogeneous, and dynamic systems. To address these limitations, microbiome engineering based on **synthetic biology** is increasingly recognized as a way to give host plants PGP advantages [26]. This approach allows laboratory selection of microbes according to their ability to colonize plants, specifically on the basis of how well they can deliver PGP advantages. Researchers could potentially deliver these microbes to specific plant species and locations (e.g., roots, leaves) at different growth and developmental stages under various environmental conditions. In addition, diverse PGP traits could be consolidated in the engineered microbiomes.

The use of genetically modified microorganisms (GMMs) is strictly regulated. However, since the first field trial for evaluating genetically modified *Pseudomonas syringae*, which was carried out on University of California experimental plots in 1987, academia and industry have investigated GMMs for nearly three decades without notable accidents and/or environmental concerns [10–12,27,28], suggesting that GMMs might be safely used under current regulations. These successes are now motivating further exploration of GMM use directly in the environment, and some US agencies have initiated research programs to develop tools and methodologies to control, counter, and even reverse GMM effects. Several companies are also investigating the use of GMMs to improve human health and agricultural sustainability, as well as related biocontainment strategies, as a core business model (e.g., Synlogic, Pivot Bio, JOYN Bio, NOVOME Biotechnologies, 64-X). In the near future, these efforts may prove microbiome engineering to be a safe strategy to increase sustainability in agriculture. Accordingly, we review here recent advances in synthetic biology and strain engineering to make microbiome engineering more amenable to agricultural applications, and discuss ways to mitigate environmental impacts (Figure 1).

Engineering Approaches for Phytomicrobiomes

Phytomicrobiomes can be engineered bottom-up or top-down, as illustrated in Figure 2. In the bottom-up approach, microbes associated with particular plant species, strains, or organs are isolated from environmental microbiomes [29,30]. After being **genetically engineered** to carry desired traits, these core microbes are reassembled as synthetic microbial communities (SynComs) [31]. Plants are then inoculated with the engineered strains, which can robustly recolonize their hosts. In the top-down approach, **horizontal gene transfer** (HGT) is used to introduce desired traits into a broad range of hosts *in situ*. One top-down strategy is to incorporate **mobile genetic elements** (MGEs), which transfer and integrate exogenous genes into a random subpopulation of microbiomes to allow holistic study of PGP traits. Another top-down strategy is to develop bacteriophage (phage) systems to engineer or eliminate particular species within populations, which allows their roles to be studied. In this section we discuss both the bottom-up and top-down approaches (Figure 2).

Bottom-Up Approach

Most synthetic biology efforts focus on established model microbes such as *Escherichia coli*. To discover more potentially novel microbial functions, non-model microbes could be directly engineered, but this is challenging because organism-specific nuances hinder the use of universal genetic tools. Use of broad host-range (BHR) plasmids is one strategy for engineering non-model microbes [32]. For example, BHR plasmids were used to study microbe–microbe interactions mediated by synthetic quorum-sensing circuits [33,34] and to elucidate the function of secondary metabolite **biosynthetic gene clusters** (BGCs) providing plant-benefiting traits [35,36]. However, although BHR plasmids are versatile, selective pressure is necessary to maintain them.

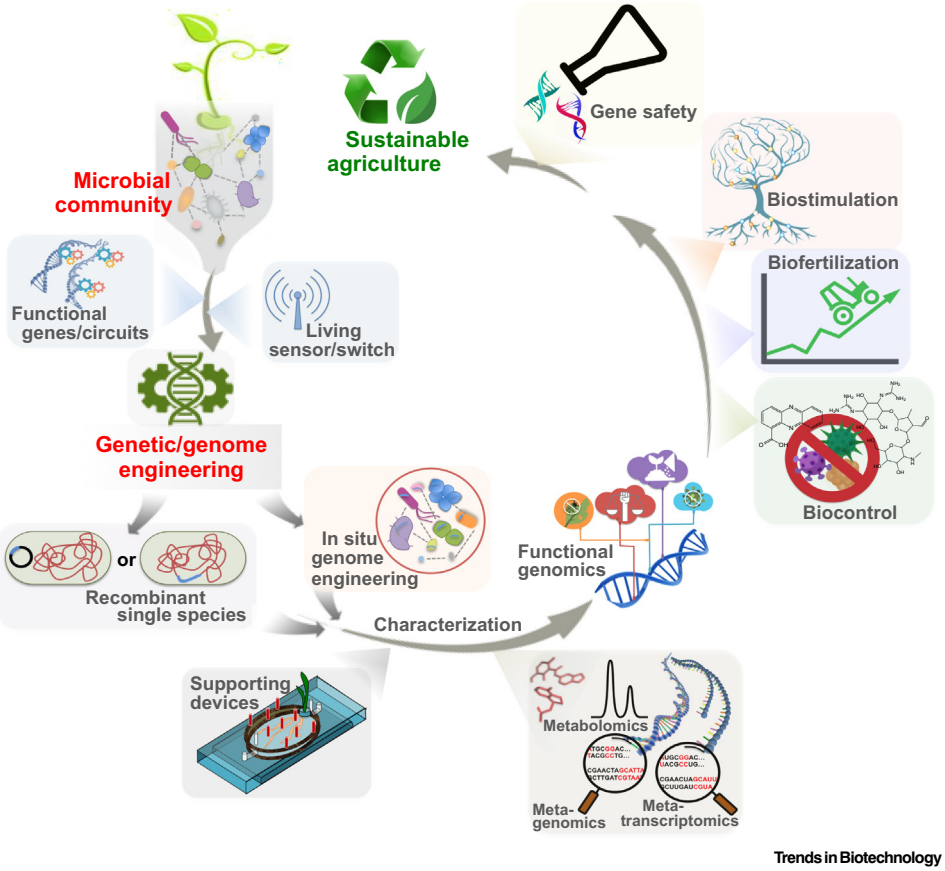

Figure 1. Overview of Synthetic Biology Enabled Microbiome Engineering in Sustainable Agriculture. Plants are associated with diverse microbes that have a range of capabilities. Some microbes can provide plant growth-promoting (PGP) advantages for their host plants; others can robustly colonize them. Both groups are important to collect in the first step. The first group provides a source of PGP genes and pathways, as well as sensors and switches to control gene expression. The second group may provide ideal chassis to deliver engineered PGP traits to host plants. Bottom-up and top-down genetic/genome engineering approaches and tools that facilitate the integration of PGP genes and pathways into rhizobacteria are discussed in the section on Engineering Approaches for Phytomicrobiomes. Types of PGP traits (biocontrol, biofertilization, and biostimulation), as well as devices combined with meta-omic strategies to study the efficacy of genetically modified microorganisms (GMMs) with engineered PGP traits, are discussed in the section on Applications of Microbiome Engineering for Plant Growth Promotion. Strategies to safeguard GMMs for field studies and applications are covered in the section on Biosafety, Biosecurity, and Biocontainment.

Microbiome engineering, conversely, requires genome-level engineering to stably maintain PGP traits. Fortunately, the ability to engineer non-model microbes has greatly improved. Figure 3 shows BHR **genome engineering** strategies that are useful for non-model microbes, including phage integrases, integrative and **conjugative** elements (ICEs), chassis-independent recombinase-assisted genome engineering (CRAGE), and others [37]. All these systems allow single-step integration of large DNA constructs >50 kb in length, and therefore permit stacking of multiple PGP traits. System selection may depend on the target bacterial phyla. The phage integrase, ICE, and CRAGE systems are more commonly used to engineer Actinobacteria, Firmicutes, and Proteobacteria, respectively.

Phage Integrase System

Phage integrases catalyze efficient recombination between phage and host attachment sites (e.g., *attP–attB*) [38]. These systems offer versatile and efficient genome engineering tools

Glossary

Biosynthetic gene cluster (BGC): a set of functionally related genes clustered near to each other that code for a specialized metabolite. Typically, one BGC is involved in production of a single compound or several similar compounds.

Clustered regularly interspaced short palindromic repeats (CRISPR): a family of DNA sequences in a prokaryote genome. These sequences, derived from DNA fragments of bacteriophages that had previously infected the prokaryote, are used to detect DNA from similar bacteriophages during subsequent infections.

Conjugation: a type of horizontal gene transfer between bacterial cells in which genetic material (such as a mobile plasmid) in the donor cell is transferred to the recipient cell by direct cell-to-cell contact.

Cre recombinase: a tyrosine integrase derived from P1 bacteriophage that catalyzes site-specific recombination between two DNA recognition sites (*loxP* sites).

Functional genomics: a field of molecular biology focused on understanding the complex relationship between genotype and phenotype on a genome-wide scale by investigating a range of processes such as transcription, translation, and epigenetic regulation.

Genetic/genome engineering: genetic engineering involves the manipulation of an organism's genes using recombinant DNA technologies. Genome engineering is a type of genetic engineering in which DNA is inserted, deleted, modified, or replaced in the genome of an organism.

Heterologous expression: recombinant DNA technology-supported expression of a gene or gene cluster in a host organism that does not naturally have that gene or gene cluster.

Horizontal gene transfer (HGT): delivery of genetic material between organisms in ways other than 'vertical' transmission of DNA from parent to offspring.

Landing pad (LP): a system that uses synthetic DNA components to facilitate gene integration.

***loxP* sites:** consist of two 13 bp palindromic sequences that flank an 8 bp spacer region. The products of Cre-mediated recombination at *loxP*

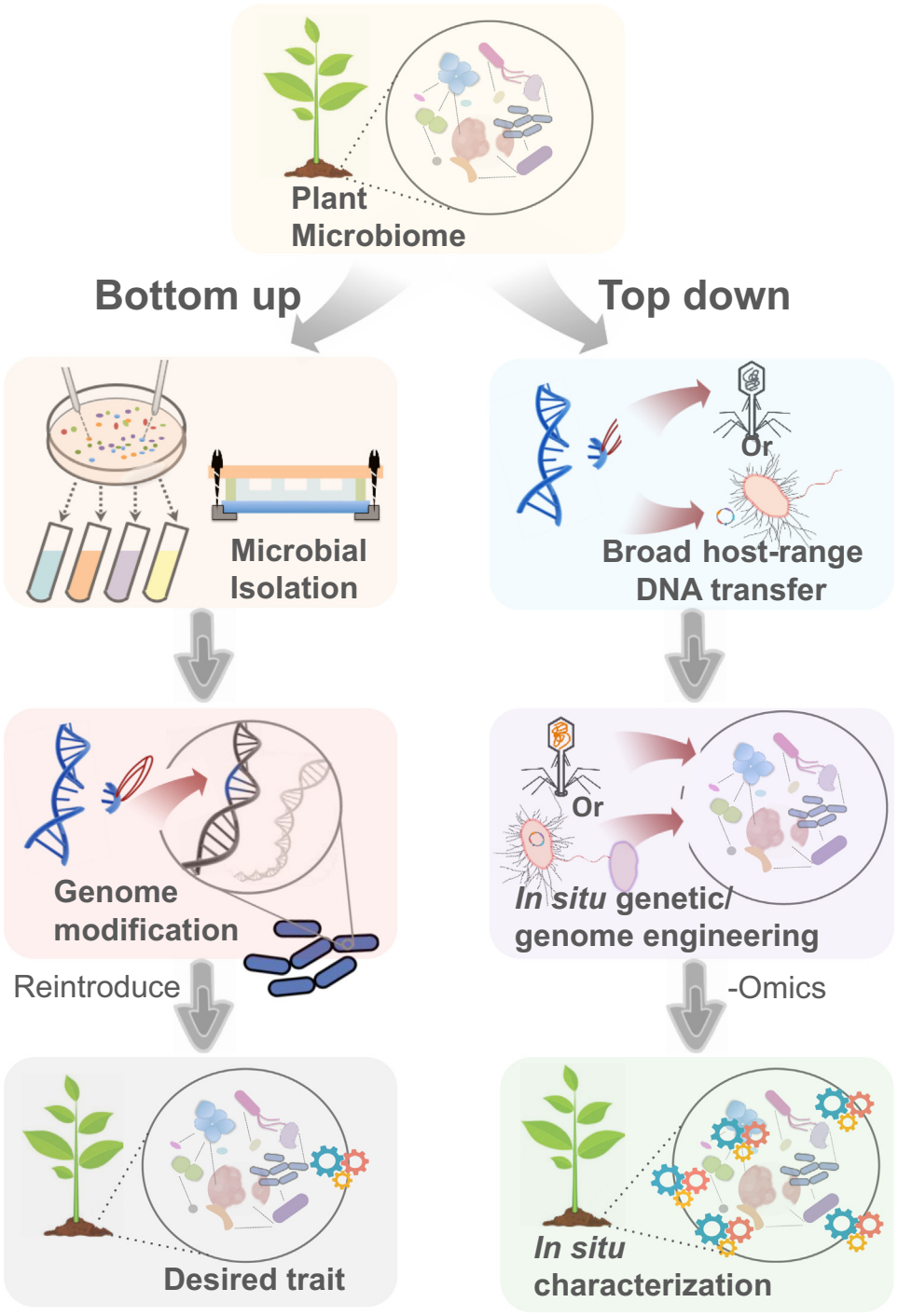

Figure 2. Approaches for Genetic/Genome Engineering of Phytomicrobiomes. There are two approaches for genetic/genome engineering of phytomicrobiomes. The bottom-up approach isolates plant-associated microbes and modifies individual strains for desired traits, then inoculates plants with the modified strains. The top-down approach uses horizontal gene transfer to introduce traits into a broad range of hosts *in situ*, and then determines their phenotypes by using supporting devices and omic technologies.

sites depend on the location and relative orientation of the *loxP* sites. Two separate DNA species both containing *loxP* sites can undergo fusion as the result of Cre-mediated recombination.

Microbiome engineering *in situ*: allows genetic payloads to be delivered to different species in a microbiome by synthetically modified phages and conjugative donors rather than by stable integration of a specific microbe.

Mobile genetic element (MGE): a type of genetic material found in all organisms that can move around within a genome or be transferred from one species or replicon to another.

Phytomicrobiome: microorganisms (including bacteria, fungi, viruses, protozoa, and Archaea) that are associated with a plant host. These microorganisms live on or inside nearly every part of the host.

Plant growth-promoting (PGP) traits: distinctive activities of some plant-associated microbes that increase host productivity or induce host resistance to pathogens or insects. PGP effects can be categorized as biocontrol, biofertilization, and biostimulation.

Synthetic biology: a field of biological engineering that aims to design, construct, or modify biological parts and modules (including genes, enzymes, pathways, and organisms) for new abilities to solve problems in medicine, manufacturing, and agriculture.

Synthetic circuit: a modular design of a combination of DNA components that can include biosensors, inducible promoters, operators, and other elements. Resembling electronic circuits, synthetic circuits enable tunable expression of gene cluster pathways, and gene transcription and translation in response to environmental stimuli.

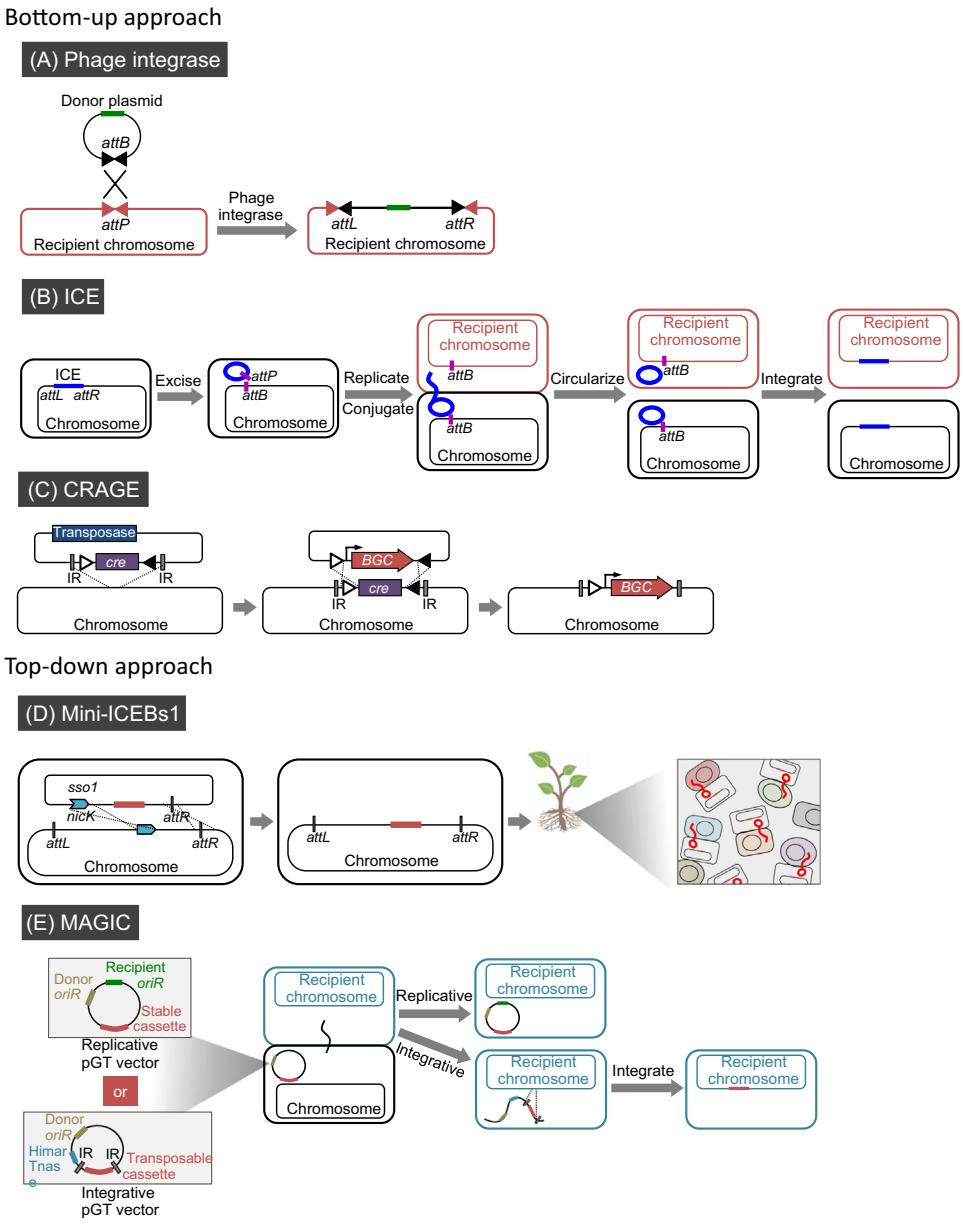

Figure 3. Broad Host-Range (BHR) Genome Engineering Strategies. (A) Phage integrase system. Phage integrase catalyzes recombination between a phage *attB* site on a plasmid and an *attP* site in the recipient bacteria [38]. (B) The integrative and conjugative element (ICE) system. ICEs in the donor chromosome flanked by *attR* and *attL* sites are excised to yield a circular plasmid. This plasmid is conjugally transferred to the ICE-free recipient cells and integrated into the recipient chromosome at an *attB* site [56]. (C) The chassis-independent recombinase-assisted genome engineering (CRAGE) system. A transposon containing a landing pad (LP) is first integrated into the recipient cells. The LP contains a *cre* recombinase gene flanked by mutually exclusive *lox* sites. The genes of interest, flanked by the same *lox* sites, are subsequently integrated into the LP on the recipient chromosome, mediated by Cre recombinase [60]. (D) Mini-ICEbs1 system. An integrative plasmid with homology to mini-ICEbs1 containing the desired DNA is transformed into the donor cell, where it integrates via homogenous recombination into mini-ICEbs1 to generate the donor cell with desired DNA [58]. The ICE donor can transfer desired DNA to undomesticated strains in the environment. (E) The metabolomics alteration of

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(Figure 3A). The *Streptomyces* phage πC31 system is widely used for functional characterization of various secondary metabolite BGCs and is compatible with diverse Actinobacteria species [39–42]. A phage Bxb1 system and intN2 integrases were used as reliable integration tools for *Pseudomonas putida* [43] and human commensal *Bacteroides* species [44–46], respectively. These systems require a native attachment site specific to each integrase, but these sites can be introduced into the host chromosome [47]. With these systems, a vector backbone used for DNA assembly remains as a scar [38]. Mini-Tn7 is another promising phage transposon-based genome engineering tool in diverse bacterial species [48–50]. However, the mini-Tn7 system can only integrate relatively small constructs [51].

ICE System

ICEs are a diverse group of MGEs that can integrate into a host chromosome via an integrase and propagate through host replication (Figure 3B). They encode functional conjugation systems to mediate their intercellular transfer [52] and autonomous replication [53]. Some ICEs integrate into conserved genes (e.g., *prfC*, tRNA genes) to increase their host range, but have evolved a seamless mechanism of integration into the 5′ end of the gene to prevent disruption [54,55]. Other ICEs are less site-specific (e.g., AT-rich regions), potentially challenging host fitness by disrupting physiologically important genes [56]. Genes encoding integrases, conjugative machinery, and transposases associated with MGEs can be identified and incorporated into domesticated mating partners, such as *E. coli* and *Bacillus subtilis* [44,57,58]. ICEs have been identified in diverse bacterial species [59]. However, their recipients are usually limited, and integration efficiency drops exponentially as DNA construct size increases [58].

CRAGE System

Yoshikuni and colleagues recently developed CRAGE, a technology that enables highly accurate and efficient integration of large, complex biological systems into chromosomes of non-model bacteria (Figure 3C) [60]. The process begins with integration of a **landing pad** (LP) containing a phage P1 ***Cre* recombinase** flanked by mutually exclusive ***loxP* sites**. Constructs with heterologous genes and pathways are then assembled into accessory vectors. As proof of concept, the group domesticated 25 diverse γ-Proteobacteria from 11 different genera by integrating a unified LP that enabled efficient integration of large BGCs. CRAGE substantially increased successful BGC expression by harnessing the native regulatory and physiological diversity of each species. CRAGE was then extended to 40 species, including α-, β-, and γ-Proteobacteria and several Actinobacteria. CRAGE's versatility makes it useful for directly engineering PGP traits into plant microbe genomes and for characterizing potential PGP gene and pathway roles *in situ*.

Overcoming restriction/modification is a common problem among these three genome engineering systems. Unique restriction/modification systems associated with target bacteria protect them from foreign DNA, potentially limiting DNA transformation. Riley and colleagues overcame this limitation by helping foreign DNA to evade the immune system of *Clostridium thermocellum* [61]. A methylome study identified multiple restriction/modification systems, and corresponding DNA methyltransferases were cloned into an *E. coli* strain to mimic the *C. thermocellum* methylome. Plasmids propagated in this strain were efficiently transformed into *C. thermocellum*.

gut microbiome *in situ* conjugation (MAGIC) system. MAGIC is used to transfer replicative or integrative pGT vectors from an engineered donor strain into amenable recipients in a complex microbiome. Replicative vectors feature a BHR origin of replication (*oriR*), whereas integrative vectors contain a transposable Himar cassette and transposase (Tnase) [77]. Abbreviations: BGC, biosythetic gene cluster; IR, inverted repeat.

Although these three bottom-up genome engineering systems offer versatile and efficient ways to engineer rhizobacteria, their throughput is limited – only several dozen microbes can be engineered at once. In addition, because many thousands of bacterial species associate natively with plants, the engineered SynComs may be rapidly diluted by resilient pre-existing microbiomes [62,63]. More knowledge of interactions among engineered microbes, pre-existing microbiomes, and host plants will be necessary for developing a robust strategy to deliver engineered PGP traits to host plants.

Top-Down Approach

A new frontier in synthetic biology offers an alternative to the bottom-up approach – **microbiome engineering *in situ*** – to recode the metagenome and build desired synthetic communities instead of only modifying specific microbes (Figure 2). *In situ* approaches enable the introduction and/or deletion of target functions into and/or out of native microbial communities with minimal disruption to context [64].

Using MGEs for In Situ Microbiome Engineering

Bacterial community members undergo abundant HGT [65] mediated through various MGEs, which can be redesigned as *in situ* genome engineering tools. In several studies, a conjugal donor strain transiently introduced into microbiomes efficiently transferred target genes to diverse microbial species across 11 different phyla in those communities [66,67]. Based on the ICE from *B. subtilis*, Brophy and coworkers created a system of miniaturized ICEs (mini-ICEBs1) that allows heterologous DNA delivery into the chromosomes of a wide range of non-model Firmicutes collected from both humans and soil, with variable efficiency (10^{-1} to 10^{-7} conjugations per donor) (Figure 3D) [58]. They also demonstrated that a *B. subtilis* donor system (XPORT) could deliver a 10 kb nitrogen fixation BGC to a synthetically defined soil microbial consortium *in situ*, and there was successful transfer to four out of six strains [58]. Other PGP advantages can also be integrated into phytomicrobiomes and delivered to targeted plant hosts this way.

Using Phages for In Situ Microbiome Engineering

In addition to MGEs, phages are ideal candidates for transducing DNA fragments because they can inject their own genomes into host chromosomes [68]. Phages can be repurposed to selectively eliminate specific pathogenic strains from the phytomicrobiome population or to transfer target PGP genes to them [69–72]. Indeed, the discovery of novel plasmids, transposable elements, and phages with various host ranges will tremendously advance synthetic biology and therefore advance more complex *in situ* manipulations of plant microbial communities [64,73,74]. In addition, tunable regulatory systems (e.g., promoters) with defined host specificities will be useful for controlling **synthetic circuit** activities across diverse plant microbial species [75,76].

New Approaches for In Situ Microbiome Engineering

Wang and colleagues reported a tool called 'metabolomics alteration of gut microbiome *in situ* conjugation' (MAGIC) based on the IncPa family RP4 conjugation system (Figure 3E). MAGIC delivers a programmed function across a microbial population with high specificity and efficiency [77]. First applied to the mouse gut, MAGIC rapidly modified the genomes of 297 native gut bacterial species (an estimated 5% of the microbiome). These genetically modified strains were isolated and reintroduced into their original community. However, because transconjugants disappeared from the population within 72 h, the stability of the system needs to be improved. In addition, donor-strain dosage could be tailored to consider recipient-specific properties and desired functions [64]. In addition, the isolation of genetically

tractable representatives (such as active recipients that are prone to acquire foreign DNA) from diverse phytomicrobiomes could expand the repertoire of new microbial chassis for applications in synthetic biology.

In both bottom-up and top-down approaches, engineered traits may burden metabolism and reduce the ability of the host to compete with other microbes. Therefore, careful assessment of PGP advantages versus potential negative impacts on plant colonization is needed. In the next section we review PGP advantages and methods to evaluate the effects on plant hosts and on the colonization efficiency of engineered bacteria.

Applications of Microbiome Engineering for Plant Growth Promotion

Phytomicrobiomes have evolved many traits important for their relationships with plants. Discovering and characterizing those traits will expand the available strategies. Recent advances in meta-omic and computational tools [78,79] have tremendously improved our understanding of microbiome diversity, niche-specific distribution, the roles of plant hosts, and the genes responsible. Genome-wide **functional genomics** is now a prominent strategy for systematic exploration and discovery of novel gene functions in microbiomes [80–83]. For instance, several secondary metabolite BGCs have been identified and some have been functionally characterized [84–87]. Using this knowledge, researchers can now design microbes that can improve crop productivity and yield through engineered functions such as biocontrol, biofertilization, and biostimulation. This section also reviews ways to test engineered functions before field studies.

Genome-Wide Functional Genomics

Near-term applications for microbiome engineering include genome-wide functional genomics. For this, transposon mutagenesis followed by sequencing (TnSeq) and **clustered regularly interspaced short palindromic repeats** (CRISPR)-based technologies are prominent strategies [82,88]. TnSeq simultaneously measures the phenotypes of many thousands of different transposon-based loss-of-function mutants grown together [89]. In several studies, coupling TnSeq with a random DNA barcode for each mutant (RB-TnSeq) helped to assess genome-wide sequence-to-function relationships for multiple microbes under diverse conditions [80,90]. This approach identified 115 genes in a model root-colonizing *Pseudomonas simiae* that are required for maximal competitive colonization of plant roots [81]. Another genome-wide genotype–phenotype mapping strategy is based on CRISPR. CRISPR-enabled trackable genome engineering (CREATE) was developed to link each guide RNA with homologous repair cassettes that also serve as barcodes to track genotype–phenotype relationships in *E. coli* and yeast [82]. More recently, Lian and coworkers established a multifunctional genome-wide CRISPR system to trifunctionally perturb the expression of genes in yeast by combining activation, interference, and deletion [91].

Biocontrol

Engineered bacteria might be useful to diagnose plant physiological changes caused by biotic stresses and to deliver desired traits [92]. Using RNA interference (RNAi), a GMM produced double-stranded RNA to silence targeted genes of a pathogen [93]. These GMMs are often used to study traits that affect specific members or a whole community predictably [94]. *Burkholderia ambifaria* is a plant pathogen, but also has biocontrolling activity via cepacin A production. In several studies, deletion of the virulence factor made *B. ambifaria* an effective biocontrol agent [95,96]. Thaxtomin, produced by the plant pathogen *Streptomyces scabiei*, is the main active ingredient of a commercial herbicide. In one study, expression of a thaxtomin biosynthesis pathway in *S. albus* J1074 increased

thaxtomin A yield 10-fold over wild-type yield [97]. Greunke and coworkers **heterologously expressed** an *ehp* BGC from *Serratia fonticola*, demonstrating production of a novel phenazine that might be a fungicide [98]. Many other systems can be repurposed via microbiome engineering to combat diverse plant pathogens, including bacterial type IV–VI secretion systems [99,100], *Bacillus thuringiensis* (Bt) toxins [101], and *Photorhabdus*/*Xenorhabdus* Cry toxins [102,103].

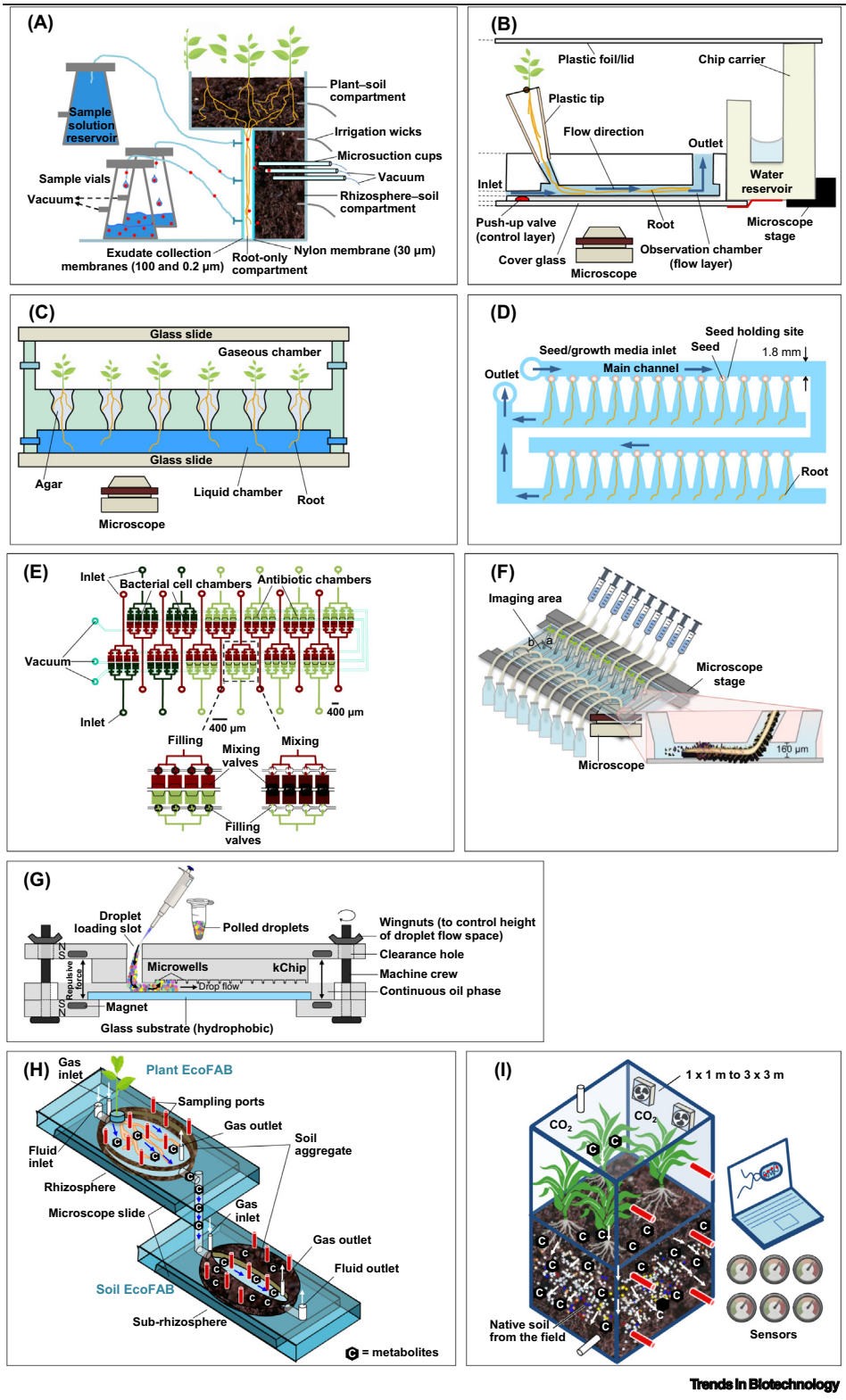
Biofertilization

Plants require macronutrients such as nitrogen (N) and phosphorus (P). In nature, plants source N from ammonia produced by nitrogen-fixing bacteria. Nitrogen fixation involves nitrogenase, an enzyme complex coded by *nif* gene clusters, whose composition varies between species [104]. Voigt and colleagues refactored and synthesized a *nif* gene cluster comprising over 20 genes (23.5 kb) and demonstrated functional expression of nitrogenase in *E. coli* [105,106]. More recently, the group transferred natural *nif* gene clusters between diverse species of rhizobia, allowing a cereal crop to fix nitrogen. Regulatory control of *nif* transcription was replaced by synthetic, genetically encoded sensors responding to natural root exudates, chemicals released by soil bacteria, and agricultural biocontrol agents [107]. Yang and colleagues explored a 'fuse-and-cleave' virus-derived polyprotein strategy [108]. Regrouping 14 essential *nif* genes into five giant genes, the group showed optimal nitrogenase activity and supported diazotrophic growth of *E. coli*.

P exists in the field in the form of inorganic or organic phosphates, but plants do not assimilate either form readily [109]. Phosphate provided as fertilizer immediately forms salts with divalent cations and is adsorbed to soil minerals [110,111]. Both plants and phytomicrobiomes excrete organic acids to solubilize phosphate and make P available to them. Soil and plants also accumulate phosphate as a form of phytate, which can be hydrolyzed to release phosphate, catalyzed by phytase [110,112]. Eighty-two sequentially diverse phytase genes across three phytase families have been synthesized [112]. Subsequent **heterologous expression** of these genes in three rhizobacteria identified several phytases with high activity for phytate hydrolysis, and this conferred a significant advantage for the growth of *Arabidopsis thaliana* with phytate as the sole phosphate source [112]. This study demonstrated that microbiome engineering can generate phytomicrobes with phosphate-solubilizing capabilities as biological alternatives to costly and environmentally damaging phosphate fertilizers.

Biostimulation

Many plant-associated microbes can synthesize plant hormones such as auxin, ethylene, and cytokinins that have crucial multifaceted roles in plants [113]. Recent work has shown that pathways for these hormones can be engineered for expression in other species. Auxins, primarily indole acetic acid (IAA), regulate most plant growth and development. Heterologous expression of the IAA synthesis pathway in *Bacillus* sp. significantly increased IAA production [114]. By integrating a quorum-sensing (QS) circuit with the IAA synthesis pathway, Zúñiga and coworkers demonstrated IAA production induced with QS in a rhizobacterium, *Cupriavidus pinatubonensis* [115]. The plant hormone ethylene leads to plant growth inhibition. 1-Aminocyclopropane-1-carboxylate (ACC) deaminase can catalyze degradation of the ethylene precursor ACC and thereby reduce ethylene production [115]. ACC deaminase expressed in the banana endophytes *Enterobacter* sp. E5 and *Kosakonia* sp. S1 promoted the growth of banana plants and increased resistance to *Fusarium* wilt [116]. Cytokinin involvement in fungal tolerance has been reported by Trdá and coworkers [117], although the complete mechanism is unknown. Moreover, some bacteria can produce abscisic acid and gibberellins; inoculation with these microbes helps plants to resist stresses [118,119].



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Methods To Evaluate the Effects of Engineered Microbiomes

Some devices allow evaluation of GMM function before field studies. For instance, lithographic 3D printing has enabled the creation of microscopic containers for organizing multiple bacterial species in essentially any 3D geometry. Their interactions can be studied and compared with those of native strains at the microscopic level [120]. Other devices provide accurate sampling methods that are indispensable for monitoring rhizosphere processes. A root exudate collector (Figure 4A) allows non-destructive sampling from soil-grown plants [121]. Many microfluidic platforms can be used for live imaging or high-throughput phenotyping of engineered root-bacteria growth and metabolism [122,123] including RootChip (Figure 4B) [124], RootArray (Figure 4C) [125], PlantChip (Figure 4D) [126,127], and static droplet array (Figure 4E) [128,129]. A microfluidic device named 'tracking root interactions system' (TRIS), that was designed to track root–bacteria interactions (Figure 4F), is also useful for studying the engineered bacteria [130]. Kehe and colleagues recently introduced kChip (Figure 4G), a platform that is capable of

Figure 4. Platforms for Phytomicrobiome Applications. (A) Rhizobox with root exudate collector. The rhizobox contains a plant–soil compartment, a rhizosphere–soil compartment, and a root-only compartment [154]. For *in situ* collection of root exudates, a sampling solution is circulated through rhizosphere–soil and root-only compartments. The microsuction cup sampling strategy is used to collect unaltered root exudates from soil-grown roots while minimizing solute transfer from the adjacent soil compartment [121]. (B) RootChip can be used for live-cell imaging, exudate metabolomic sampling, and rapid modulation of environmental conditions. Roots and seedlings grown on solid agar medium in pipette tips are plugged into access holes and mounted on the RootChip's observation chambers with liquid medium. The entire RootChip can be mounted on a microscope stage using the chip carrier and enclosed to maintain humidity. Once the root tip enters the observation chamber, the setup is connected to a pressure line with a computer-controlled actuated valve system and transferred to a suitable inverted microscope for wide-field fluorescence or confocal observation. The device can be mounted vertically or horizontally [124]. (C) RootArray to monitor spatiotemporal gene expression dynamics in plant cells. RootArray is a higher-throughput model of RootChip, with 64 wells filled with agar. The seeds are manually planted in the wells, and the chip is sealed by two glass slides at both top and bottom surfaces. Growth media are then injected and continuously exchanged by a peristaltic pump. The roots can be monitored in both vertical and horizontal positions by automated imaging systems for reconstructing the 3D shape of each root and live imaging the gene expression in individual cells [125]. (D) PlantChip for high-throughput phenotyping of plant and microbial interactions. The PlantChip is made of a series of funnel-shaped microchannels to automatically trap seeds at the narrow top opening of the funnel by hydrodynamic fluid loading. This avoids damage to the seeds during positioning or manipulation. The roots elongate from the seeds through the seed-holding site toward the bottom site of the tapered microchannel. The observation chamber is vertical, enabling continuous monitoring of the whole plant [126]. (E) Static droplet array for quantifying on-chip polymicrobial interactions and the susceptibility of bacteria to antimicrobials in polymicrobial cultures. This platform consists of a control layer for actuating the mixing and filling valves, and a fluidic layer that contains the flow channels and 48 wells (4.8 nl each) filled with dyed aqueous solutions. A close-up 48-well array can be used to combinatorially screen for various microbial activities such as antibacterials (green solutions) against polymicrobial bacterial cells (red solutions). These solutions can be mixed (dark-red solution) [129]. (F) The tracking root interactions system (TRIS) for tracking root–bacteria interactions. The TRIS device allows monitoring of individual plant roots in nine separate channels. Each channel contains independent inlet and outlet ports to prevent cross-contamination between channels during bacterial inoculation. A third port, at one end of the channel, is used for the introduction of the germinated plant root. This setup the dynamic behavior of bacteria at and around the root surface to be captured under controlled conditions with high spatial and temporal resolution [130]. (G) kChip within a loading apparatus and droplet loading procedure. This apparatus consists of an acrylic housing and hydrophobic glass substrate. The kChip naturally forms a seal with the top piece of acrylic. In its unclamped state, a flow space is maintained between 500 and 700 μm by a repulsive magnetic force such that droplets can flow under the kChip. Tilting the apparatus moves droplets through the flow space, and random sets of droplets spontaneously group within microwells [131]. (H) EcoFAB as a standardized fabricated ecosystem. EcoFABs are constructed either through direct fabrication or through printing molds that are used to cast polydimethylsiloxane or other materials. Plants are germinated on plates and transferred to the sterilized EcoFAB, to which microbes can be added. The chamber enables control of liquid flow through the chamber, spatially defined imaging of the root system, and the ability to sample and add microbes and materials to the root system in a spatially defined manner. Destructive sampling allows analysis of microbe, root, and shoot parameters in detail [131]. (I) An overview of the EcoPOD concept. These enclosed 'pilot-scale' ecosystems can replicate natural ecosystems in a laboratory setting with regard to temperature, humidity, and other important climatic parameters, with precise, fine-scale environmental sensors. Prototype EcoPODs can range from 1 to 3 m^3 in a closed environment that allows longer-term control, manipulation, and real-time imaging, sample collection, and data integration of replicated plant–soil–microbe–atmosphere interactions (https://ecopods.lbl.gov/publications).

rapid, massively parallel, bottom-up construction and screening of synthetic microbial communities [131].

A controlled laboratory habitat termed 'EcoFABs' was developed for mechanistic studies in desired environmental conditions (Figure 4H) [132]. This system enables dynamic and detailed investigation of plant and plant–microbe interactions, plant growth, root morphology, exudate composition, and microbial localization. Pilot-scale growth-chamber 'EcoPODs' have also been developed; these enclosed environments of several cubic meters allow direct and intensive monitoring and manipulation of replicated plant–microbiome–ecosystem interactions over the complete plant life cycle under conditions relevant to the real world (https://ecopods.lbl.gov/publications). EcoPODs can control temperature, humidity, and other important climatic parameters, and can monitor soil moisture, oxygen, and nutrients; sensor outputs can be integrated using computer models to gain coherent understanding of the EcoPOD environment. Multiple EcoPODs allow larger-scale evaluation of plant–microbe interactions (Figure 4I). Both EcoFABs and EcoPODs help to accelerate the translation of engineered PGP bacteria to field applications.

Biosafety, Biosecurity, and Biocontainment

Environmental concerns about releasing GMMs must be addressed. Several US agencies have initiated programs for biosafety, biosecurity, and biocontainment strategies (Obama White House, 2015; Safe Genes program, Darpa, 2017) to increase understanding of how synthetic DNAs and GMMs behave under various environmental conditions and across diverse organisms for multiple generations. Biosafety and biosecurity concerns also include HGT of synthetic DNA and the emergence of microbes harmful to the environment that are difficult to eliminate. Tracking strategies are being developed. For instance, several DNA watermarks have been independently devised for DNA coding regions, regulatory sequences, and noncoding DNA sequences to encrypt information by the DNA-Crypt algorithm [133,134].

In addition, systems to effectively contain GMMs (<1 in 10^8 cells escape) in various environments must be developed [135–138]. Some systems are designed to control cell viability in a defined environment via auxotrophy, essential gene regulation including minimal genomes, and toxin expression [137,139–141]. For instance, to restrict the viability of GMMs to media containing synthetic small molecules, Gallagher and colleagues have developed riboregulators that tightly control the expression of essential genes and a nuclease-based addiction module that cleaves the host genome (Figure 5A) [142]. In addition, reported kill switches of 'essentializer' and 'cryodeath' circuits (type II TA system CcdB–CcdA) employ a bistable cI/Cro memory switch and a toxin-expressing cold-inducible promoter, respectively (Figure 5B) [143]. Other synthetic kill switches are 'Deadman' and 'Passcode' (Figure 5C) [144]. The Deadman switch uses unbalanced reciprocal transcriptional repression to couple a specific input signal with cell survival. The Passcode switch uses a similar two-layered transcription design and incorporates hybrid LacI–GalR family transcription factors for diverse and complex environmental inputs. Related safeguard systems can be readily reprogrammed for other environmental inputs, regulatory architecture, and mechanisms of killing [137].

If they escape, these microbes must be detected and effectively countered. Cell-to-cell communication systems mediated by signaling molecules could be engineered as living sensors to enable recipient-specific and population-level control [33,145,146]. GMMs can be effectively countered through, for example, antimicrobial secondary metabolites and type IV–VI secretion machinery (discussed in the section on Biocontrol). Notably, phages are another promising system that could be engineered to deliver CRISPR [147], anti-CRISPR [148–150], LshC2c2 [151],

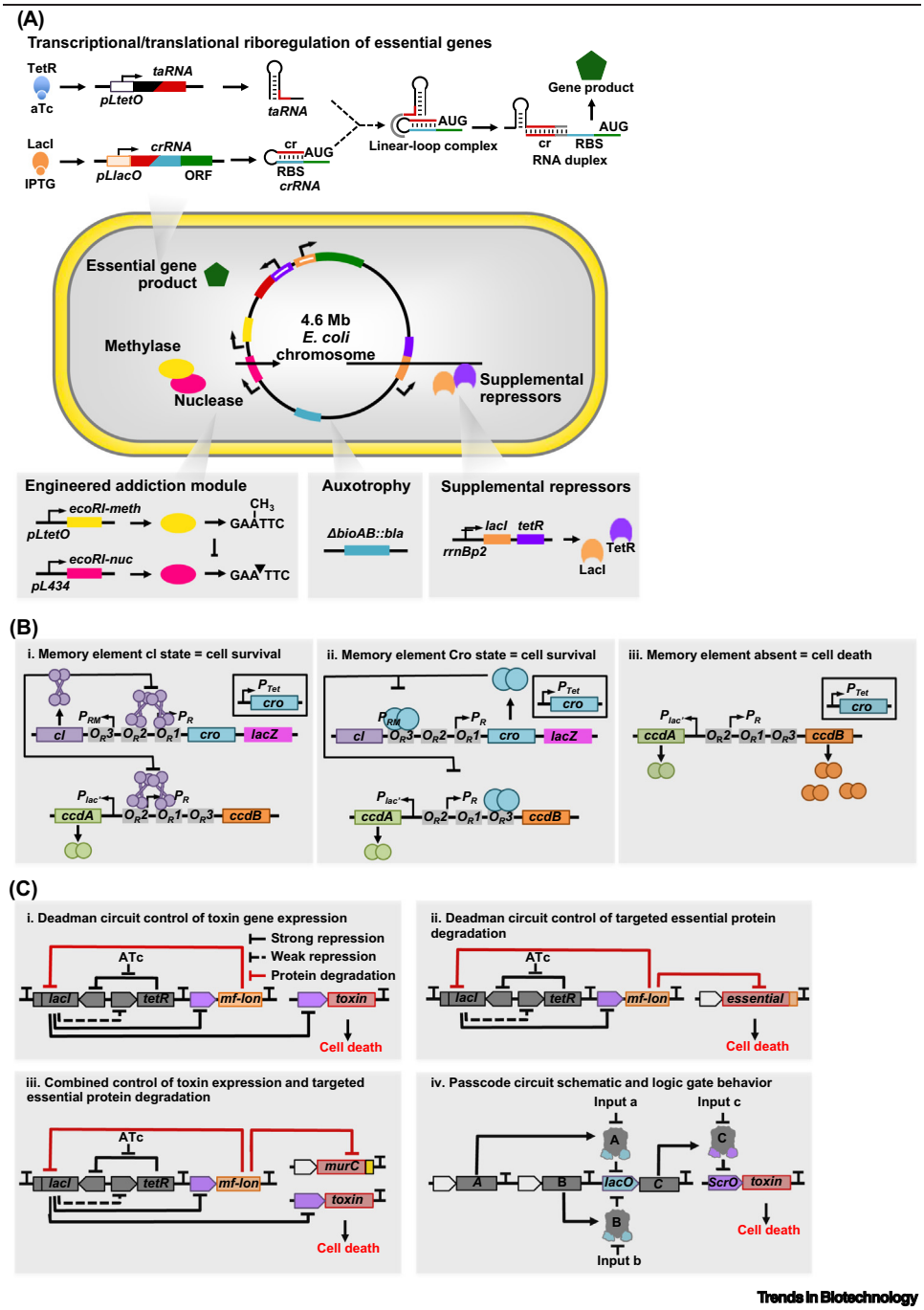

Figure 5. Highlighted Strategies for Biocontainment. (A) Design of multilayered genetic safeguards: riboregulation, engineered addiction, auxotrophy, and supplemental repressors. Riboregulation system: a *pLtetO* promoter, that is repressed by TetR and induced by aTc, drives *trans*-activating RNA (taRNA); a *pLlacO* promoter, repressed by LacI and induced by IPTG, drives *cis*-repressed RNA (crRNA) and an essential gene. crRNA and taRNA fold through a linear loop intermediate to reveal the crRNA ribosome-binding site, which permits expression (green). Supplementary TetR (purple) and LacI (green) are constitutively expressed from the genome. The carbenicillin resistance gene (*bla*) replaces *bioAB*, resulting in biotin autotrophy (blue). Constitutive EcoRI nuclease (nuc; magenta) enables inducible cell killing in the absence of EcoRI methylase

(Figure legend continued at the bottom of the next page.)

and sOPTiKO/sOPTiKD systems [152] tailored to control functions of synthetic DNAs and viability of GMMs [147,153]. Future systems may include multiple layers of these technologies to synergistically reduce the rate of GMM escape.

In the US, the Environmental Protection Agency (EPA), Food and Drug Administration (FDA), and the Department of Agriculture regulate genetically modified organisms. The EPA regulates pesticidal GMMs under the Federal Insecticide Fungicide and Rodenticide Act (FIFRA) and the Federal Food Drug and Cosmetic Act (FFDCA). The EPA also regulates GMMs with biofertilization activities under the Toxic Substances Control Act (TSCA) [28]. The aforementioned tracking, safeguarding, and countering systems are exciting new directions in synthetic biology. In theory, they may offer safe and effective strategies for environmental use of GMMs. However, these systems require further GMM modification, which will create more challenges under current regulations. As these systems become more technologically mature and stable, a new regulatory framework may be needed to assess their risks separately from the risks of engineered PGP activities.

Concluding Remarks and Future Perspectives

Field application of PGP microbes may be a promising strategy for sustainable agriculture, but success has been variable, likely because of varying environmental conditions, poor microbial colonization, and limited persistence in the rhizosphere. **Genetic/genome engineering** of robust root colonizers or colonization with large subpopulations of phytomicrobiomes may help to overcome these limitations. Recent advances in synthetic biology are enabling non-model microbes to be engineered at even the subpopulation level *in situ*. The next step is to assess the persistence of GMMs in association with plants and the efficacy of engineered PGP advantages for disease control and crop yields in greenhouses, pilot plots, and demonstration fields before commercial adoption. In particular, the environmental impacts of field treatments with GMMs must be evaluated over lengthy periods (see Outstanding Questions).

Genome-wide functional genomics increasingly allows discovery of new genes that are responsible for PGP activities including biocontrol, biofertilization, and biostimulation. Increased exploration of plant microbiome engineering to exploit these gene functions is expected. Application of available *in situ* monitoring and ongoing improvement are important first steps to address the unknowns in this complex network. There is a need for global meta-omic approaches for quantifying all possible changes, as well as integrative models to interpret meta-omic datasets, and these capabilities are continuously improving. Although ethics, regulations, and public perception require continuing global discussion and consensus, new initiatives that develop strategies for biosafety,

(meth; yellow), which is controlled by aTc [142]. (B) (i) Expression of cI represses the expression of *cro* and *lacZ* in the memory element, while simultaneously repressing the expression of *ccdB* in the essentializer element. *ccdA* is expressed at a constitutive low level. (ii) Exposure to tetracycline leads to a pulse of expression of *cro* from the trigger element; expression of Cro allows expression of *lacZ* while simultaneously repressing *cI* and *ccdB*. (iii) Memory element is absent. Without repression from cI or Cro, *ccdB* is expressed at lethal levels [143]. (C) Deadman and passcode kill switch. (i) Additional palindromic *lacI* operator sites are included in the toxin gene promoter to minimize leaky toxin expression, and a transcriptional terminator is introduced upstream of the promoter to insulate the gene from spurious transcription. Removal of the survival signal (ATc) increases Deadman-induced cell death. (ii) Inclusion of the *mf-Lon*-specific pdt#1 tag on the specified essential gene causes mf-Lon-mediated degradation of the essential protein upon Deadman circuit activation. (iii) Combined control of toxin expression and targeted essential protein degradation increases Deadman-induced cell death. (iv) Passcode circuit schematic and logic gate behavior. Cell survival requires the continued presence of inputs (i) and (ii), and the absence of input (iii). The loss of inputs (i) or (ii), or the addition of input (iii), causes the passcode circuit to activate toxin expression, leading to cell death [144]. Abbreviations: aTC, anhydrotetracycline; IPTG, isopropyl β-thiogalactopyranoside; ORF, open reading frame; RBS, ribosome binding site.

Outstanding Questions

How can we incorporate recent advances in multi-omic and computational tools, even artificial intelligence and machine learning, to expedite and maximize the discovery of new genes and gene clusters encoding PGP traits in plant-associated microorganisms and microbiomes?

How can we develop new and stable tools and strategies for genetic and genome engineering of a broader range of hosts? Can we learn more about how to use genome and metagenome dataspaces to explore useful genetic tools?

Is it possible to standardize and modularize genetic and genome engineering tools for more accurate genetic and genome engineering? How can we standardize strategies to evaluate and tailor alternative genetic tools for defined microbe species?

How can we precisely control the expression of defined genes and gene clusters to desired levels in synthetic microbes?

What is the best way to achieve stable maintenance of PGP traits in engineered plant microbes in various environments? Can we maintain the survival and reproduction of synthetic microbes in actual field environments?

How can we improve *in situ* exploration of the functions of microbes on plants? How can we improve the devices and strategies used for *in situ* sampling and characterization of microbial functions on plants?

There is a need to expand genome-wide genotype–phenotype mapping and create comprehensive and diversified genomic libraries of organisms composing phytomicrobiomes. How can we improve genome-wide functional genomics to exploit the functions of mined genes and gene clusters?

How best can we accelerate the translation of engineered PGP bacterial traits to applications in the field?

How can genetically modified microbes be safely used in sustainable agricultural development? Can we accelerate the development

biosecurity, and biocontainment may lead to a tipping point, after which GMMs can be safely used in the development of more sustainable agriculture.

of tools and strategies for biosafety, biosecurity, and biocontainment before applying genetically modified microbes to the environment?

Can public perception of genetically modified microbes be improved?

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