

Genetic code expansion enables plant-directed control of bacterial activity

Corresponding Author: Professor Jennifer Brophy

Parts of this Peer Review File have been redacted as indicated as we could not obtain permission to publish the reports from one of the peer reviewers.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I read with great interest the manuscript entitled "Genetic code expansion enables plant-directed control of bacterial activity" and found the work to be creative, technically rigorous, and conceptually innovative. The central concept—using a plant-biosynthesized noncanonical amino acid as an orthogonal communication channel to control bacterial behavior via genetic code expansion—is elegant and genuinely novel. The authors have done something that few papers achieve: they have connected three fields (GCE, plant synthetic biology, agricultural biotechnology) in a convincing, experimentally grounded way.

The manuscript is also refreshingly honest about limitations. The authors quantify OMY exudation (3.1 μM), report toxicity (IC₅₀ 94–101 μM), test and rule out two mechanisms, and then engineer conditional expression to mitigate fitness costs. The soil experiments and hairy root transformations in tomato and poplar demonstrate extensibility beyond idealized lab conditions. This is a foundation paper, not an overhyped claim of field readiness, and I appreciate that restraint.

I do, however, have several concerns that should be addressed before publication. None are fatal, but collectively they will improve the paper's precision, accessibility, and long-term utility. For example, the work is convincing in agar, hydroponics, and controlled soil experiments, but field robustness is still an open question.

Major comments

1. Terminology: "non-standard" vs. "non-canonical" amino acids

Throughout the manuscript, the authors use the term "non-standard amino acid (nsAA)" to describe OMY and related compounds. By current conventions in the genetic code expansion field, this terminology is imprecise. The term canonical refers specifically to the 20 (+2) amino acids that are universally incorporated into proteins by the translational machinery. Amino acids not used in standard ribosomal translation—including OMY, but also naturally occurring metabolites such as citrulline and ornithine—are more appropriately classified as non-canonical amino acids (ncAAs). The term "non-standard" is ambiguous, since many compounds outside the canonical set are nevertheless standard components of cellular metabolism.

More importantly, this distinction is relevant to the manuscript's central theme of orthogonality. The term "ncAA" explicitly emphasizes the translational context and immediately conveys to specialist readers that the orthogonality being discussed arises from genetic code expansion rather than from simple metabolic novelty. I therefore recommend replacing "nsAA" with "ncAA" throughout the manuscript (or introducing "non-canonical amino acid" at first use and subsequently using the abbreviation "ncAA").

2. The OMY concentration threshold requires more explicit discussion

The authors report 2-fold induction at 1 μM OMY and 5-fold at 10 μM . Root exudate from constitutive lines averages 3.1 μM —just above the 2-fold threshold. Root-cap specific lines produce 0.73 μM , which is below the 2-fold threshold in bulk exudate, yet you still detect spatial luminescence. This is encouraging but requires a more careful discussion of: (i) Whether bulk exudate concentration underestimates local rhizosphere concentration (likely yes, due to root-surface gradients); (ii) What concentration bacteria actually experience at the root surface versus in bulk soil; (iii) Whether field soil (with OMY-

binding particles or degrading microbes) would reduce effective concentration

Please add a paragraph in the Discussion acknowledging that while the system works in controlled conditions, translation to non-sterile field soils will require either (a) higher OMY production, (b) more sensitive ncAA incorporation systems, or (c) both.

3. The toxicity mechanism is unresolved but you imply more than the authors demonstrate

They rule out two mechanisms (precursor depletion, misincorporation) convincingly. However, the current text gives readers no sense of what might be happening. The authors don't need to solve it, but they should offer testable hypotheses.

Possibilities include: (i) OMY acting as a competitive inhibitor of aromatic amino acid transporters; (ii) OMY interfering with auxin biosynthesis or signaling (consistent with root gravitropism phenotype); (iii) OMY affecting cell wall crosslinking (tyrosine derivatives are precursors for lignin and suberin).

Even a single sentence naming plausible directions would strengthen the paper and guide future work.

4. Strain-specific differences in amber codon optimization

The manuscript finding that UD1022 (the wild isolate) performs optimally with one amber codon while PY79 requires two is important and should not be buried. This suggests that amber suppression efficiency, background termination fidelity, or ncAA transport differs between strains. Please add a sentence to the Discussion acknowledging that platform optimization may be strain-specific and that field deployment may require re-optimization for target bacterial strains.

5. Minor overclaim in the abstract

Line 25: "non-standard amino acids (nsAA) as a platform for plant-based control" — Consider revising to "as a proof-of-concept platform" or simply "as a tool". The term "platform" implies a degree of generalization and robustness that you demonstrate for multiple species but not yet for multiple ncAAs or multiple target genes in realistic environments. This is a small point, but precision here will protect you from overly critical remarks.

6. Broader conceptual context

The manuscript is framed primarily through the lens of genetic code expansion and plant synthetic biology. However, its broader significance extends beyond protein engineering toward orthogonal biological communication and engineered ecological interactions.

The creation of a plant-to-bacterium communication channel mediated by a non-canonical amino acid represents a particularly interesting example of ecological engineering enabled by genetic code expansion. Therefore the authors are encouraged to expand the Discussion and place the work in the context of emerging literature on orthogonal biological systems (e.g. ACS chemical biology 13 (4):854-870), ecological applications of genetic code expansion in the context of synthetic biology (e.g. Frontiers in Synthetic Biology 1:1255472), and biocontainment strategies based on expanded genetic codes (e.g. ACS Synthetic Biology, 13(9), 2992-3002 (2024)). Such a discussion would help readers appreciate that the importance of this work lies not only in ncAA incorporation itself, but also in the creation of a programmable information channel between distinct biological partners.

Minor comments

7. Line 26: "biosynthesis" → "biosynthesize" (verb form)

8. Line 84: "OMY exhibits minimal phytotoxicity" — This is relative to meta-tyrosine and canavanine, but your own data show clear toxicity at 100 μ M. Consider "reduced phytotoxicity compared to known allelopathic ncAAs"

9. Line 170: The heading "O-methyl-L-tyrosine effects on plant growth" — The compound is already abbreviated as OMY. Use "OMY effects on plant growth" for consistency.

10. Line 247: "plant growth-promoting bacterium" — This is a claim about UD1022 that you do not test directly in this paper (you cite Bishnoi et al. 2015). That's fine, but consider "putative plant growth-promoting bacterium" or simply "wild soil isolate UD1022, previously reported to promote plant growth"

11. Figures: Extended Data Fig. 5 is critical for field relevance. Consider moving it to a main figure, or at least citing it more prominently in the Results section.

12. Terminology consistency: as already discussed, the authors use "non-standard," "non-proteinogenic," "non-canonical," and "non-native" interchangeably. Standardize on non-canonical amino acid (ncAA) throughout, with "non-proteinogenic" only where biosynthetic origin (not translational inactivity) is the relevant distinction.

Overall assessment

This is a high-quality, creative manuscript that deserves publication in a top-tier journal after revision. Your use of GCE as an enabling technology—rather than as the object of study itself—is exactly the kind of translational application the field needs. The work will interest plant biologists, synthetic biologists, and agricultural engineers. It also points, intentionally or not, toward a future in which engineered orthogonal communication channels reshape ecological interactions.

I look forward to seeing the revised manuscript.

Recommendation: Minor Revision

Confidence: High (the science is sound; the requested revisions primarily involve clarification, contextualization, and improved framing rather than additional experimentation).

Reviewer #2

(Remarks to the Author)

The manuscript by Zhong et al. describes a novel approach to engineer plants and bacteria to allow specific interaction between the two organisms whereby molecules synthesized by the plant control gene expression in the bacteria. This approach introduces GCE (genetic code expansion) in *Bacillus* bacteria, which enables gene expression in these bacteria to be dependent on a non-standard amino acid (nsAA), o-methyl tyrosine (OMY), which only engineered plants are capable of synthesizing. GCE has been previously demonstrated in bacteria, however Zhong et al. used it in a very creative manner to establish the required nsAA to be produced by plants, enabling a dedicated interaction between the plant and the microbe. The authors demonstrated convincingly that engineered bacteria growing in the plant rhizosphere can only express a gene of interest (e.g., reporter genes) when the plant synthesizes and secretes a specific nsAA. This work represents an exciting advance in the fields of synthetic biology and plant-microbe interactions, as *Bacillus subtilis* bacteria typically establish beneficial associations with plants, and future developments of this technology could enable bacteria growing in the soil to provide essential nutrients to plants, for example.

The manuscript is well written, and the experiments are well designed. Most results are clearly shown, with a few exceptions being when plant fluorescence results were shown; these are mentioned below and should be addressed. The conclusions are supported by the experimental results, and the statistical analyses seem appropriate for the experimental design used. This work should be of interest to a broad audience.

One minor concern about any future practical applications of this technology relates to the fact that OMY over-production (using the Ubiquitin promoter) was shown to have some toxicity in plants, with significantly reduced root growth. The authors circumvented this toxicity in part by restricting OMY synthesis to the root cap or by controlling it with a chemically inducible promoter. However, the authors did not show whether these alternative approaches, which result in much lower concentrations of OMY produced by the plant, are still sufficient to control gene expression in the bacteria. From a practical point of view, root-cap-restricted OMY synthesis would be preferable, although overall OMY concentrations in this case are very low. Testing whether this limited production of OMY still results in controlled gene expression in the bacteria would significantly enhance the impact of this work.

A few specific comments are provided below to improve some figures and clarify some information provided in the text.

Text:

The authors could comment on whether it was already known that OMY, when engineered to be synthesized by plants, is secreted by the roots, as this was not explicitly shown here. Was this a fortuitous outcome, it had been demonstrated before, or did the authors test this?

It's not clear how estradiol was applied to induce MfnG expression in plants. Why don't we see luminescence in the shoots in Fig. 4h?

In the Methods section, describe how plants were co-cultivated with the bacteria on agar. This is important to understand how the plant roots are able to interact with the bacteria. Are the plant roots laid on top of the agar that already contains the bacteria? Or are the roots growing into the agar containing the bacterial cells? From the images, it appears that the bacteria are present in a thin layer of top agar that is laid on top of the plant roots. In any case, it is important to provide a description of these methods.

Lines 813-816: How old were the seedlings when the bacteria were added?

Line 843: *Agrobacterium tumefaciens* GV 3101 harboring the desired plasmids....

Figures:

In Figure 2c, the plants shown appear to only have cotyledons. It would be better to show older plants that contain true leaves, e.g., at 14 days. The fluorescence signal is very difficult to see, at least in the submitted file. Perhaps add a brightfield image of the plants as well.

In Figure 2e, replace it with the images of luminescence present on the whole plate from the side-by-side plate (shown in Extended Data Fig. 2c), because Fig. 2e doesn't really show what's described in the text (Lines 160-163). The text describing Extended Data Fig. 2c (Lines 165-167) should then refer to Fig. 2e.

Line 201: the described results actually are shown in Extended Data Fig. 4f (not Extended Data Fig. 4e).

Line 202: "Yet, exogenous OMY treatment of wild-type seeds had minimal effect on germination" – This is 4e. Panels should be flipped in Extended Data Fig. 4.

In Figure 4a, fluorescence signal located in root caps is a little difficult to see. Would it be possible to show an overlay of the GFP on the brightfield image?

Similarly, in Figure 4b, the image appears very dim (at least in my copy), and it is difficult to see GFP expression throughout the plant.

In Extended Data Fig 2d (described in Lines 228-232), luminescence in the central panel is very difficult to see. Interestingly, it's easier to see in the composite image.

In Extended Data Fig. 5e-g, it's not clear that the purple bars (or histograms) represent co-cultivation of UD1022 with wild-type Arabidopsis plants. Perhaps add a fourth row on the x-axis in (f) and (g) to indicate wt plants?

In Fig. 5c (and Extended Data Fig.6), why is the luminescence brightest at the root tips if MfnG is ubiquitously expressed? Discuss the reason(s) for this observation.

In line 289, this should be Extended Data Fig. 7.

Figure 1 legend: describe other genetic components, pVeg, pSer, etc., shown in panel b.

Extended Data Fig. 5 legend, Line 635: 1 day is shown in panel b, and 3 days is shown in panel c.

Minor typos:

Line 264: delete "next"

Figure 6 legend, "R. rhizogenes transfers its plasmids into tomato cells,...", it should say T-DNA, not plasmids.

Line 676, in Methods: rifampicin concentration is missing.

Reviewer #3

(Remarks to the Author)

Zhong et al. demonstrated that non-standard amino acids can be used as a platform for plant-based control of microbial activity. Using genetic code expansion, they engineered O-methyl-L-tyrosine (OMY)-dependent control of protein expression in *Bacillus subtilis*. They then stably incorporated OMY biosynthesis into *Arabidopsis thaliana* and evaluated OMY phytotoxicity. Given the detrimental impacts of constitutive OMY biosynthesis on *A. thaliana*, the authors utilized tissue-specific and conditional production of OMY to reduce fitness costs while maintaining plant-based bacterial control. The authors also tested OMY-based bacterial control using composite tomato and poplar plants to demonstrate that their system can be extended to other plant species. While the overall study is useful and expands the current repertoire of signaling molecules for modulating plant-bacterial interactions, the results are mainly descriptive and qualitative. Additional quantification beyond visual luminescence intensity scales and better spatial resolution would be helpful to understand how effective OMY-based control is in co-cultivation systems. The value of this system compared to other molecules such as rhizopines, sucrose, and opines appears to be the potential orthogonality of non-standard amino acids for targeted plant-bacteria communication. However, this was not clearly demonstrated in relevant conditions including the use of non-sterile soils or in the presence of multiple bacterial species to understand off-target effects. The major emphasis of the paper appears to be engineering OMY biosynthesis in plants and controlling its phytotoxicity. Much more supporting evidence and additional mechanistic validation would be needed to strengthen the authors' claims. Therefore, I do not think that this manuscript is acceptable for publication in *Nature Biotechnology*. My other questions are listed below.

1. The authors investigated the effects of OMY on phytotoxicity, but what about the effects of OMY on bacteria? Is there a concentration dependent effect that could impact bacterial fitness? Forti et al. (2025) report that OMY can be consumed by other bacteria while Zer et al. (2025) report that m-Tyr, another non-proteogenic amino acid, is highly toxic to various cyanobacteria.
2. The authors report OMY biosynthesis in the μM range ($3.1 \mu\text{M}$ from 18 ubiquitously expressing seedlings and $0.73 \mu\text{M}$ from 50 root cap-specific seedlings) after 48 hrs. In their bacterial reporter assays, they show significant fold-change in induction, but at $100 \mu\text{M}$ OMY. This raises the question of what is the actual fold-change in induction at plant-produced OMY conditions? Is the level of OMY production from plants meaningful? (See point 3)
3. The data presented are endpoint measurements taken after often 4 days of co-cultivation. Given the low concentration of OMY biosynthesized per plant and the amount of OMY needed to produce fold-changes in luminescence, reporting of earlier timepoints and the OMY concentration over time would provide a clearer picture of if this signaling process is dynamic and can be controlled. Can you achieve a range of bacterial protein production or is it just off and on with this system once a minimum threshold of OMY is reached?
4. The authors used sterile soil throughout their experiments, but it would be informative to understand how orthogonal this technology is in non-sterile soils and in the presence of other soil microbes that could consume the OMY, reducing the local concentration, or have other unintended effects.
5. It would be interesting to understand OMY distribution surrounding the plants and how that corresponds to the observed luminescence intensity and bacterial density. The claim that plant-produced OMY enables genotype-specific induction of bacterial gene expression would be strengthened if they labeled the bacteria to decouple potential localization effects from luminescence. Is stronger signal due to more bacteria being present around those plants? Is there a concentration gradient of OMY from the plant outwards that corresponds to luminescence profiles?
6. In Figures 1 and 5, the authors switch to reporting data in mM despite using μM for every other graph. It would be beneficial to stay consistent with the units to understand the magnitude of the dose.
7. Figure 4, unclear what g-h caption "event #4, event #1, right three, event #2, left three" means.

Author Rebuttal letter:

Blue denotes response to reviewers; Red denotes manuscript changes

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

I read with great interest the manuscript entitled "Genetic code expansion enables plant-directed control of bacterial activity" and found the work to be creative, technically rigorous, and conceptually innovative. The central concept—using a plant-biosynthesized noncanonical amino acid as an orthogonal communication channel to control bacterial behavior via genetic code expansion - is elegant and genuinely novel. The authors have done something that few papers achieve: they have connected three fields (GCE, plant synthetic biology, agricultural biotechnology) in a convincing, experimentally grounded way.

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I do, however, have several concerns that should be addressed before publication. None are fatal, but collectively they will improve the paper's precision, accessibility, and long-term utility. For example, the work is convincing in agar, hydroponics, and controlled soil experiments, but field robustness is still an open question.

We thank the reviewer for their careful analysis of our manuscript and their helpful suggestions, which are all addressed in the revised manuscript and detailed below.

Major comments

1. Terminology: "non-standard" vs. "non-canonical" amino acids

Throughout the manuscript, the authors use the term "non-standard amino acid (nsAA)" to describe OMY and related compounds. By current conventions in the genetic code expansion field, this terminology is imprecise. The term canonical refers specifically to the 20 (+2) amino acids that are universally incorporated into proteins by the translational machinery. Amino acids not used in standard ribosomal translation—including OMY, but also naturally occurring metabolites such as citrulline and ornithine—are more appropriately classified as non-canonical amino acids (ncAAs). The term "non-standard" is ambiguous, since many compounds outside the canonical set are nevertheless standard components of cellular metabolism. More importantly, this distinction is relevant to the manuscript's central theme of orthogonality. The term "ncAA" explicitly emphasizes the translational context and immediately conveys to specialist readers that the orthogonality being discussed arises from genetic code expansion rather than from simple metabolic novelty. I therefore recommend replacing "nsAA" with "ncAA" throughout the manuscript (or introducing "non-canonical amino acid" at first use and subsequently using the abbreviation "ncAA").

We agree that non-canonical is a more precise/appropriate term and have updated every instance of "non-standard"/"nsAA" in the manuscript to "non-canonical"/"ncAA."

2. The OMY concentration threshold requires more explicit discussion

The authors report 2-fold induction at 1 μM OMY and 5-fold at 10 μM . Root exudate from constitutive lines averages 3.1 μM —just above the 2-fold threshold. Root-cap specific lines produce 0.73 μM , which is below the 2-fold threshold in bulk exudate, yet you still detect spatial luminescence. This is encouraging but requires a more careful discussion of: (i) Whether bulk exudate concentration underestimates local rhizosphere concentration (likely yes, due to root-surface gradients); (ii) What concentration bacteria actually experience at the root surface versus in bulk soil; (iii) Whether field soil (with OMY-binding particles or degrading microbes) would reduce effective concentration

Reviewers 2 & 3 had similar questions. To address these comments, we performed additional reporter activation experiments at lower concentrations of OMY. We used flow cytometry to sensitively measure OMY-dependent mNeonGreen fluorescence and found statistically significant activation with as little as 0.05 μM (1.07-fold) and 0.5 (7.7-fold) μM OMY for PY79. We added this data as Extended Data Fig. 4cd.

Though it is difficult to compare luminescence across assay types, there appears to be a gap between the reporter activity at OMY concentrations consistent with bulk measurement (0.7 - 3 μ M) and the luciferase signal observed in co-cultivation. We agree with the reviewer that this may be due to bulk measurements underestimating OMY concentrations at the plant root or higher bacterial cell density near the root where nutrients are abundant. We added a paragraph to the discussion that describes these possibilities and to suggest future experiments for measuring OMY with spatial precision. The new results and discussion paragraph are pasted here for ease of review:

Extended Data Fig. 4. *B. subtilis* growth and protein expression with OMY. (c-d) Fluorescence of *B. subtilis* PY79 (c) or UD1022 (d) expressing an OMY-dependent mNeonGreen reporter across a range of OMY concentrations in minimal media. Fluorescence measured via flow cytometry. Statistical significance was evaluated using a one way ANOVA with Tukey HSD posthoc test (** $p < 0.01$, **** $p < 0.0001$).

Extended Data Fig. 4c reference [lines 237 - 242]:

"Because this concentration falls below the initially characterized range (Fig. 1c), we tested incorporation at lower OMY concentrations. Using flow cytometry to sensitively measure OMY-dependent mNeonGreen fluorescence, we found induction at OMY concentrations as low as 0.05 μ M (1.67-fold) and 0.5 μ M (7.7-fold) (Extended Data Fig. 4c). Thus, the reporter remains sensitive to OMY below the 1 μ M tested with plate reader fluorescence."

Discussion [lines 345 - 355]:

"Interestingly, bulk measurements of OMY did not reflect the level of reporter activation at plant roots in co-cultivation experiments. This is likely because bulk measurements underestimate OMY concentration at the root, where bacteria contact the plant directly^{56,57}. Though OMY concentration gradients may explain the strong luminescence of OMY-dependent reporter strains grown with MfnG-expressing plants, the OMY-independent reporter also showed a modest increase in luminescence near the roots of every plant genotype, suggesting bacterial cell density may increase near the roots⁵⁸ and contribute to a local boost in luminescence signal. Though we did not directly measure OMY concentration or bacterial cell density at the root, labeling bacteria with a constitutive fluorescent marker, quantifying OMY and bacterial CFU extracted from the gel matrix at different plate locations would help measure the contribution of chemical and cell concentration gradients to reporter signals in future work."

Please add a paragraph in the Discussion acknowledging that while the system works in controlled conditions, translation to non-sterile field soils will require either (a) higher OMY production, (b) more sensitive ncAA incorporation systems, or (c) both.

We agree that translating this technology to agricultural soil will require optimization. In an agricultural setting, the engineered bacteria will face competition from resident bacteria for nutrients and OMY could be rapidly degraded. Environmental fluctuations may also threaten strain persistence and change the conditions for incorporation. We agree that greater ncAA sensitivity may make the system more robust to non-sterile conditions and we hope that future optimization would be focused on optimizing microbial incorporation at low ncAA concentrations. We are hesitant to suggest greater OMY production as a solution as this may compromise plant fitness and render the technology less useful in the field. We added a paragraph to the discussion acknowledging the challenges associated with field deployment [lines 329 - 343]:

"Future work can also optimize the system for agricultural soils, where resident bacteria and environmental fluctuations may degrade OMY, influence incorporation, and make it difficult for engineered strains to compete in the rhizosphere. Greater ncAA sensitivity, achieved through engineering of the aminoacyl-tRNA synthetase/tRNA pair for greater translation efficiency at low ncAA concentrations⁴⁹, may make signaling more robust to these fluctuations without requiring phytotoxic levels of OMY production. Engineering natural field isolates may help address colonization and competition challenges. However, we found that maximum fold-change required different amber codon configurations in PY79 and UD1022. This may reflect variation in amber suppression efficiency^{27,50,51}, background translational termination fidelity^{52,53}, or ncAA transport and availability^{54,55} between strains. Thus, field deployment may require optimization of the orthogonal translation system and the amber codon number in each bacteria with plant hosts in the target environment. Testing this system in non-sterile soils with diverse native microbial communities will also be important for evaluating potential off-target effects and confirming that OMY-based signaling remains specific to engineered strains in complex, multi-species environments."

3. The toxicity mechanism is unresolved but you imply more than the authors demonstrate

They rule out two mechanisms (precursor depletion, misincorporation) convincingly. However, the current text gives readers no sense of what might be happening. The authors don't need to solve it, but they should offer testable hypotheses. Possibilities include: (i) OMY acting as a competitive inhibitor of aromatic amino acid transporters; (ii) OMY interfering with auxin biosynthesis or signaling (consistent with root gravitropism phenotype); (iii) OMY affecting cell wall crosslinking (tyrosine derivatives are precursors for lignin and suberin). Even a single sentence naming plausible directions would strengthen the paper and guide future work.

These are great suggestions. We've expanded our discussion of OMY toxicity mechanisms in the discussion [lines 323-327]:

"Future work can explore additional hypotheses for OMY toxicity, including competitive inhibition of aromatic amino acid transporters and biosynthetic enzymes⁴⁶, interference with auxin biosynthesis or signaling (consistent with impaired root gravitropism)⁴⁷, and interference with gibberellic acid biosynthesis or signaling (consistent with reduced germination)⁴⁸, as well as methods for amelioration."

4. Strain-specific differences in amber codon optimization

The manuscript finding that UD1022 (the wild isolate) performs optimally with one amber codon while PY79 requires two is important and should not be buried. This suggests that amber suppression efficiency, background termination fidelity, or ncAA transport differs between strains. Please add a sentence to the Discussion acknowledging that platform optimization may be strain-specific and that field deployment may require re-optimization for target bacterial strains.

We agree that optimal GCE design will be strain specific. We incorporated this information into the discussion paragraph acknowledging challenges in field deployment. Relevant edits to the paragraph are underlined here [lines 329 - 343]:

"Future work can also optimize the system for agricultural soils, where resident bacteria and environmental fluctuations may degrade OMY, influence incorporation, and make it difficult for engineered strains to compete in the rhizosphere. Greater ncAA sensitivity, achieved through engineering of the aminoacyl-tRNA synthetase/tRNA pair for greater translation efficiency at low ncAA concentrations⁴⁹, may make signaling more robust to these fluctuations without requiring phytotoxic levels of OMY production. Engineering natural field isolates may help address colonization and competition challenges. However, we found that maximum fold-change required different amber codon configurations in PY79 and UD1022. This may reflect variation in amber suppression efficiency^{27,50,51}, background translational termination fidelity^{52,53}, or ncAA transport and availability^{54,55} between strains. Thus, field deployment may require optimization of the orthogonal translation system and the amber codon number in each bacteria with plant hosts in the target environment. Testing this system in non-sterile soils with diverse native microbial communities will also be important for evaluating potential off-target effects and confirming that OMY-based signaling remains specific to engineered strains in complex, multi-species environments."

5. Minor overclaim in the abstract

Line 25: "non-standard amino acids (nsAA) as a platform for plant-based control" — Consider revising to "as a proof-of-concept platform" or simply "as a tool". The term "platform" implies a degree of generalization and robustness that you demonstrate for multiple species but not yet for multiple ncAAs or multiple target genes in realistic environments. This is a small point, but precision here will protect you from overly critical remarks.

The abstract text has been revised to [lines 24-26]:

"Here, using O-methyl-L-tyrosine (OMY) as a model compound, we establish non-canonical amino acids (ncAA) as a tool for plant-based control of associated microbial activity."

6. Broader conceptual context

The manuscript is framed primarily through the lens of genetic code expansion and plant synthetic biology. However, its broader significance extends beyond protein engineering toward orthogonal biological communication and engineered ecological interactions.

The creation of a plant-to-bacterium communication channel mediated by a non-canonical amino acid represents a particularly interesting example of ecological engineering enabled by genetic code expansion. Therefore the authors are encouraged to expand the Discussion and place the work in the context of emerging literature on orthogonal biological systems (e.g. ACS chemical biology 13 (4):854-870), ecological applications of genetic code expansion in the context of synthetic biology (e.g. Frontiers in Synthetic Biology 1:1255472), and biocontainment strategies based on expanded genetic codes (e.g. ACS Synthetic Biology, 13(9), 2992-3002 (2024)). Such a discussion would help readers appreciate that the importance of this work lies not only in ncAA incorporation itself, but also in the creation of a programmable information channel between distinct biological partners.

We appreciate the suggestion to place our paper into a broader GCE context and have included the following paragraph in the discussion to do so [lines 357 - 370]:

"Here, we extend GCE technology from intracellular protein engineering to programmable interactions between organisms. Historically, GCE largely focused on engineering new chemistries within a single organism⁵⁹, including exogenous supply of synthetic ncAAs to control gene expression via translational switches^{27,60} and survival of engineered microbes^{61,62}. Pioneers in the field have long speculated that these kinds of orthogonal biological systems would improve biosafety of engineered organisms by enabling one kind of "genetic firewall" that would limit horizontal gene transfer^{63–65}. Although these tools and principles each pertain to applications in environmental biotechnology, prior work has not realized the creation of a self-sufficient pair of engineered organisms sharing a private communication channel via GCE. One exception is our own recent work where we demonstrated a proof-of-concept of ecological control of gene expression and survival among two engineered *E. coli* strains²⁵. The system that we present here represents proof-of-concept that GCE can link plants and environmentally relevant microbes across kingdoms using an ncAA as the shared signal, with autonomous and spatiotemporally controlled synthesis of the ncAA by a multicellular organism."

Minor comments

7. Line 26: "biosynthesis" → "biosynthesize" (verb form)

This sentence has been revised to [Line 27-28]:

"Then, we engineer agronomically diverse plants, including *Arabidopsis*, tomato and poplar, to biosynthesize OMY."

8. Line 84: "OMY exhibits minimal phytotoxicity" — This is relative to meta-tyrosine and canavanine, but your own data show clear toxicity at 100 μ M. Consider "reduced phytotoxicity compared to known allelopathic ncAAs"

This sentence has been revised to [Line 84-85]:

"Relative to plant-produced ncAAs that function as allelopathic compounds, such as meta-tyrosine²⁹ and L-canavanine³⁰, OMY exhibits reduced phytotoxicity."

9. Line 170: The heading "O-methyl-L-tyrosine effects on plant growth" — The compound is already abbreviated as OMY. Use "OMY effects on plant growth" for consistency.

The heading has been revised to [Line 177]:

"OMY effects on plant and bacterial growth"

10. Line 247: "plant growth-promoting bacterium" — This is a claim about UD1022 that you do not test directly in this paper (you cite Bishnoi et al. 2015). That's fine, but consider "putative plant growth-promoting bacterium" or simply "wild soil isolate UD1022, previously reported to promote plant growth"

This sentence has been revised to [Line 260-262]:

"We therefore extended the OMY platform to *Bacillus subtilis* UD1022, a wild isolate that colonizes *Arabidopsis* roots and was previously reported to promote plant defense and growth⁴¹."

11. Figures: Extended Data Fig. 5 is critical for field relevance. Consider moving it to a main figure, or at least citing it more prominently in the Results section.

As suggested, we have moved data from Extended Data Fig. 5 to main text Fig. 5. The new Fig. 5 and its caption are reproduced here for review. Changes to figure caption are underlined [Lines 692-708]:

Figure 5. OMY-dependent gene expression in a plant growth-promoting bacterium. (a) Activity of an OMY-dependent mNeonGreen reporter in *Bacillus subtilis* UD1022 across a range of OMY concentrations in minimal media. (b) Activity of NanoLuc reporters containing zero, one, or two amber stop codons in *B. subtilis* UD1022 with 0 μ M (black) or 100 μ M (purple) OMY supplementation in rich media (LB). Data are the mean of three replicates collected simultaneously from three separate cultures. Error bars show s.d. and dots are individual data points. (c-d) Representative flow cytometry histograms of 10,000 events of UD1022 strains harboring a OMY-independent mNeonGreen reporter (green) or OMY-dependent mNeonGreen reporter extracted from autoclaved soil supplemented with 0 μ M OMY (black) or 500 μ M OMY (pink). Cells were extracted from the soil 1 day (c) or 3 days (d) after inoculation. (e) Median fluorescence for the same strains and conditions as (c-d) at 1 day and 3 days after inoculation for three replicates. (f) Co-cultivation of *B. subtilis* UD1022 expressing the OMY-dependent luciferase reporter containing two amber stop codons with wild type Col-0 (top) or proUBQ10::sfGFP-MfnG (bottom) *Arabidopsis* seedlings. Composite images overlay

luminescence (10s exposure) and brightfield. Seedlings transferred to bacterial-agar matrix at 10 days post-stratification and co-cultivated for 4 days. Colorbar represents luminescence intensity (Fire LUT). Additional images in Extended Data Fig. 6. Scale bars, 1 cm.

12. Terminology consistency: as already discussed, the authors use "non-standard," "non-proteinogenic," "non-canonical," and "non-native" interchangeably. Standardize on non-canonical amino acid (ncAA) throughout, with "non-proteinogenic" only where biosynthetic origin (not translational inactivity) is the relevant distinction.

As stated above, we have revised every instance of "non-standard"/"nsAA" in the manuscript to "non-canonical"/"ncAA".

Overall assessment

This is a high-quality, creative manuscript that deserves publication in a top-tier journal after revision. Your use of GCE as an enabling technology—rather than as the object of study itself—is exactly the kind of translational application the field needs. The work will interest plant biologists, synthetic biologists, and agricultural engineers. It also points, intentionally or not, toward a future in which engineered orthogonal communication channels reshape ecological interactions.

I look forward to seeing the revised manuscript.

Recommendation: Minor Revision

Confidence: High (the science is sound; the requested revisions primarily involve clarification, contextualization, and improved framing rather than additional experimentation).

Reviewer #2 (Remarks to the Author):

The manuscript by Zhong et al. describes a novel approach to engineer plants and bacteria to allow specific interaction between the two organisms whereby molecules synthesized by the plant control gene expression in the bacteria. This approach introduces GCE (genetic code expansion) in *Bacillus* bacteria, which enables gene expression in these bacteria to be dependent on a non-standard amino acid (nsAA), o-methyl tyrosine (OMY), which only engineered plants are capable of synthesizing. GCE has been previously demonstrated in bacteria, however Zhong et al. used it in a very creative manner to establish the required nsAA to be produced by plants, enabling a dedicated interaction between the plant and the microbe. The authors demonstrated convincingly that engineered bacteria growing in the plant rhizosphere can only express a gene of interest (e.g., reporter genes) when the plant synthesizes and secretes a specific nsAA. This work represents an exciting advance in the fields of synthetic biology and plant-microbe interactions, as *Bacillus subtilis* bacteria typically establish beneficial associations with plants, and future developments of this technology could enable bacteria growing in the soil to provide essential nutrients to plants, for example.

The manuscript is well written, and the experiments are well designed. Most results are clearly shown, with a few exceptions being when plant fluorescence results were shown; these are mentioned below and should be addressed. The conclusions are supported by the experimental results, and the statistical analyses seem appropriate for the experimental design used. This work should be of interest to a broad audience.

We thank the reviewer for their careful analysis of our manuscript and their helpful suggestions, which are all addressed in the revised manuscript and detailed below.

One minor concern about any future practical applications of this technology relates to the fact that OMY over-production (using the Ubiquitin promoter) was shown to have some toxicity in plants, with significantly reduced root growth. The authors circumvented this toxicity in part by restricting OMY synthesis to the root cap or by controlling it with a chemically inducible promoter. However, the authors did not show whether these alternative approaches, which result in much lower concentrations of OMY produced by the plant, are still sufficient to control gene expression in the bacteria. From a practical point of view, root-cap-restricted OMY synthesis would be preferable, although overall OMY concentrations in this case are very low. Testing whether this limited production of OMY still results in controlled gene expression in the bacteria would significantly enhance the impact of this work.

Reviewers 1 & 3 had similar questions. To address these comments, we performed additional experiments to test reporter activation at lower concentrations of OMY. We found statistically significant activation of the OMY-dependent reporter in *B. subtilis* with as little as 0.05 μM OMY and 0.5 (7.7-fold) μM OMY for PY79. We added this data as Extended Data Fig. 4cd. Extended Data Fig. 4. *B. subtilis* growth and protein expression with OMY. (c-d) Fluorescence of *B. subtilis* PY79 (c) or UD1022 (d) expressing an OMY-dependent

mNeonGreen reporter across a range of OMY concentrations in minimal media. Fluorescence measured via flow cytometry. Statistical significance was evaluated using a one way ANOVA with Tukey HSD posthoc test (** $p < 0.01$, **** $p < 0.0001$).

We also quantified the luciferase signal in our co-cultivation experiments and found that the root cap-specific line was able to generate a ~2.2-fold increase in luciferase signal at the root relative to the midpoint between plants. This was more than when the OMY-dependent bacteria were cultivated with the wild type plants, which showed a small (1.4-fold) increase in luciferase signal (peak/midpoint intensity). We suspect that this signal is due to a combination of higher bacterial density at the root and low-level readthrough of the amber stop codons. We observed small luminescence peaks with the OMY-independent reporter at each seedling regardless of genotype (~1.2-fold above midpoint intensity), which supports the idea that bacterial density near the root can boost the local luminescence signal. Though we did not directly measure OMY concentration or bacterial cell density at the root, we added a sentence to the discussion suggesting experiments that could help measure OMY and bacterial cell concentration gradients during co-cultivation in a future study. Luminescence quantification was added to Fig. 2, Fig. 4, and Extended Data Fig. 2. The Results [lines 165 – 172, 237 – 244, 250 – 255] and Discussion [lines 345 – 355] were updated to include these points.

To make the luminescence signal easier to see, we also changed the max threshold of luminescence for co-cultivation of OMY-dependent bacteria. All of these changes are highlighted in the revised manuscript and representative figure panels/text are pasted below for review:

Panels g-i from Fig. 4 show an example of the new luminescence quantification:

Figure 4. Conditional MfnG expression reduces fitness costs while maintaining plant-based bacterial control. (g) Co-cultivation of OMY-dependent *B. subtilis* PY79 luciferase reporter strain (as in Fig. 2e) with proBRN1::sfGFP-MfnG seedlings. Scale bars, 1 cm. (h) Luminescence intensity profile across the x-axis of the proBRN1::sfGFP-MfnG plate in (g) (orange) and wild type Col-0 plate in Extended Data Fig. 2c (grey). (i,j) Fold change (i) and maximum (j) luminescence intensity for wild-type Col-0 (grey, $n=5$) and proBRN1::sfGFP-MfnG (orange, $n=5$) samples in (h). Statistical significance was evaluated using a Welch's test (** $p < 0.01$).

For comparison, here is the plate image showing luminescence signal from wild type Col-0 plants co-cultivated with OMY-dependent bacteria using the new luminescence scale (10,000 signal threshold). This is a panel from Extended Data Fig 2c:

Results section referencing the new figure panels [lines 237 - 246] (changes underlined):
“We next tested whether conditional expression produces sufficient OMY for bacterial control. LC-MS analysis of conditioned media detected OMY in proBRN1::sfGFP-MfnG samples, though at lower concentrations than proUBQ10::sfGFP-MfnG (0.73 μM from 50 root cap-specific seedlings versus 3.1 μM from 18 ubiquitously expressing seedlings cultured in 12 mL media), which is consistent with BRN1 expression being restricted to ~19% of root cells^{38,40}. Because this concentration falls below the initially characterized range (Fig. 1c), we tested incorporation at lower OMY concentrations. Using flow cytometry to sensitively measure OMY-dependent mNeonGreen fluorescence, we found induction at OMY concentrations as low as 0.05 μM (1.67-fold) and 0.5 μM (7.7-fold) (Extended Data Fig. 4c). Thus, the reporter remains sensitive to OMY below the 1 μM tested with plate reader fluorescence. In co-cultivation assays, proBRN1::sfGFP-MfnG plants activated OMY-dependent NanoLuc expression ~2.2-fold above midpoint intensity (Fig. 4g-j, Extended Data Fig. 2c). Though luminescence was lower than observed with proUBQ10::sfGFP-MfnG plants, peaks at individual plant locations remained well defined.”

Discussion [lines 345 - 355]:

“Interestingly, bulk measurements of OMY did not reflect the level of reporter activation at plant roots in co-cultivation experiments. This is likely because bulk measurements underestimate OMY concentration at the root, where bacteria contact the plant directly^{56,57}. Though OMY concentration gradients may explain the strong luminescence of OMY-dependent reporter strains grown with MfnG-expressing plants, the OMY-independent reporter also showed a modest increase in luminescence near the roots of every plant genotype, suggesting bacterial cell density may increase near the roots⁵⁸ and contribute to a local boost in luminescence

signal. Though we did not directly measure OMY concentration or bacterial cell density at the root, labeling bacteria with a constitutive fluorescent marker, quantifying OMY and bacterial CFU extracted from the gel matrix at different plate locations would help measure the contribution of chemical and cell concentration gradients to reporter signals in future work.”

A few specific comments are provided below to improve some figures and clarify some information provided in the text.

Text:

The authors could comment on whether it was already known that OMY, when engineered to be synthesized by plants, is secreted by the roots, as this was not explicitly shown here. Was this a fortuitous outcome, it had been demonstrated before, or did the authors test this?

Previous work found tyrosine in the root exudate of several plant species (McLaughlin et al., 2023). We hypothesized that OMY would similarly be secreted and believe our experiments demonstrating OMY accumulation in hydroponic media support this hypothesis. We edited a sentence in the Results [lines 145 – 147] to include these points (change underlined):
“We then transformed *Arabidopsis thaliana* Col-0 with proUBQ10::sfGFP-MfnG via floral dip to generate stable transgenic lines, hypothesizing that biosynthesized OMY could be exuded because tyrosine and many other amino acids are commonly found in root exudate³⁶.”

With the added citation: McLaughlin S, Zhelnina K, Kosina S, Northern TR, Sasse J. The core metabolome and root exudation dynamics of three phylogenetically distinct plant species. *Nature Communications* (2023) 14:1649.

It's not clear how estradiol was applied to induce MfnG expression in plants. Why don't we see luminescence in the shoots in Fig. 4h?

β -estradiol was mixed into the bacteria-agar matrices prepared for plant-bacteria co-cultivation. We expect that β -estradiol was primarily absorbed through the roots, which are in direct contact with the media and lack the protective cuticle of aerial tissues, making them comparatively more amenable to solute uptake. It has been demonstrated that β -estradiol also does not move systemically (root-to-shoot) in plants (Zong et al., 2017). Thus, production of MfnG-GFP is expected to be highest in roots in our assays. The fluorescence images in Fig. 4b are consistent with this expression pattern. Expression of MfnG-GFP in the roots would contribute to higher OMY concentrations and stronger luciferase signal near the roots. We clarified the β -estradiol application method in the Results and Methods sections. The relevant text is copied here for review:

Results (changes underlined) [lines 248 - 250]:

“We applied β -estradiol at the start of proXVE::sfGFP-MfnG plant co-cultivation with OMY-dependent bacteria by mixing it into the agar as it cooled.”

Methods (changes underlined) [lines 1179-1193]:

“To create the bacterial-agar matrix for co-cultivation of plants and bacteria, *B. subtilis* strains were streaked from frozen glycerol stocks onto LB agar plates with appropriate antibiotics and incubated at 37 °C overnight. Colonies were inoculated into 50 mL LB without antibiotics in 200 mL flasks and incubated at 37 °C with 250 rpm shaking. After cultures reached OD₆₀₀ ~1 (3-4 h), they were decanted into 50 mL centrifuge tubes (Corning 352070) and washed three times with 25 mL sterile 1X PBS (Sigma-Aldrich P4417-100TAB), each time pelleting at 4000 rpm for 10 min (ThermoScientific Sorvall X4R Pro TX1000 rotor). The final pellet was resuspended in 1 mL 1X PBS. MS agar media (0.5X MS basal salts, 1% sucrose, 1% agar, 0.05% MES, pH 5.7) was prepared, autoclaved, and allowed to cool to ~55 °C. Bacteria were then mixed into the media at a final OD₆₀₀ of 0.1. When appropriate, β -estradiol was mixed into the MS agar at this stage (1:1000 v/v) to a final concentration of 20 μ M. After mixing, the bacteria-agar mixture was immediately pipetted into sterile petri dishes. For *Arabidopsis* seedlings, 30 mL of mixture was added to 100 mm square plates (Genesee 26-275); for tomato composite hairy roots, 50 mL to 120 mm square plates (Greiner Bio-One 688161); for poplar composite hairy roots, 250 mL to 245 mm square plates (Corning 431111).”

In the Methods section, describe how plants were co-cultivated with the bacteria on agar. This is important to understand how the plant roots are able to interact with the bacteria. Are the plant roots laid on top of the agar that already contains the bacteria? Or are the roots growing into the agar containing the bacterial cells? From the images, it appears that the bacteria are present in a thin layer of top agar that is laid on top of the plant roots. In any case, it is important to provide a description of these methods.

In our co-cultivation assays, plants were laid on the surface of solidified agar. This agar contains bacteria, which are mixed in as the agar is cooling, prior to pouring the plates. Thus, the roots rest on top of the bacteria-agar matrix during the co-cultivation period.

The thin layer visible in the images is agarose mixed with luciferin. This luciferin-agarose mixture is applied on top of the co-cultivated roots and bacteria solely to deliver the luciferase substrate for imaging. The overlay does not contain any bacterial cells.

We have revised the Methods text to better describe the assay. We specify that plant roots are placed on the surface of the bacteria-agar matrix and clarify that the luciferin-agarose film is added at the imaging step to deliver the luciferin substrate. We have also added a reference to Supplementary Fig. 1 earlier in the text, at the point where plant placement is first described, to assist the reader in understanding our assay. These changes were made in the Methods section titled “Co-cultivation of plants and bacteria on plates for luciferase imaging of OMY incorporation” (changes underlined) [lines 1195 - 1218]:

“Once the bacteria-agar matrix cooled, plants were transferred onto the plates for co-cultivation using sterile forceps. Plant roots were placed directly onto the surface of the solidified bacteria-agar matrix, allowing root-bacteria contact to occur at the agar surface. Arabidopsis plants or composite plants were placed onto each plate, which were then sealed with micropore tape, wrapped in foil from the bottom up to the top of the shoot, and returned to the growth chamber for co-cultivation in standard conditions. A schematic of the co-cultivation setup is provided in Supplementary Fig. 1.

After 4 days of co-cultivation (5 days for poplar), luciferin was applied to the plate as an agarose film. This luciferin-agarose mixture is overlaid on top of the co-cultivated roots and bacteria to deliver the luciferase substrate for imaging. To create this film, 0.8% agarose (Research Products International, A20090) was dissolved in water and cooled to ~55 °C. NanoFuel (NanoLight Technology 324-50, Arabidopsis assays) or NanoGlo (Promega N1110, tomato and poplar assays) substrates were diluted 1:25 into their respective buffers, then added to the molten agarose solution for a final dilution of 1:500. Luciferin-agarose mixtures were immediately applied to co-cultivation plates by slowly pipetting a thin surface layer over plants roots and bacteria (10 mL for Arabidopsis assays, 15 mL for tomato, 60 mL for poplar). Co-cultivation plates were immediately imaged with an iBright Imaging System (Invitrogen FL1500) with the following settings: brightfield (Membrane channel, auto-exposure), luminescence (ChemiBlot channel, 30s exposure unless otherwise indicated), fluorescence (RFP Ex: 515-545nm, Em: 568-617nm, 15ms exposure; GFP Ex: 455-485nm, Em: 508-557nm, 15ms exposure). All image processing was performed using the Fiji software. Processing steps include: setting thresholds for maximum brightness, applying LUTs (Green for green fluorescence, Fire for luminescence), generating colorbars, and merging channels.”

Lines 813-816: How old were the seedlings when the bacteria were added?

This sentence has been revised to [lines 999-1000]:

“500 µL of the cell solution was added to the soil containing 11 day old seedlings”

Line 843: *Agrobacterium tumefaciens* GV 3101 harboring the desired plasmids....

This sentence has been revised to [lines: 1029 – 1031]:

“*Agrobacterium tumefaciens* GV3101 cultures harboring the desired plasmids were inoculated from frozen glycerol stock (18% glycerol) into 14 ml culture tubes (VWR 47729-566) containing 2 mL of LB media with antibiotics.”

Figures:

In Figure 2c, the plants shown appear to only have cotyledons. It would be better to show older plants that contain true leaves, e.g., at 14 days. The fluorescence signal is very difficult to see, at least in the submitted file. Perhaps add a brightfield image of the plants as well.

We updated Fig. 2c to a more mature plant (proUBQ10::sfGFP-MfnG, 11 days post-stratification). We also included a brightfield overlay (right) and a zoom in of the primary and lateral root tips to see fluorescence. The figure panel and caption are reproduced here for review [lines 615-618]:

Figure 2. Heterologous MfnG expression enables OMY biosynthesis in planta and plant-directed control of bacterial gene expression. (c) sfGFP expression in homozygous proUBQ10::sfGFP-MfnG seedlings 11 days post-stratification. Widefield fluorescence microscopy (500 ms exposure, left) and composite brightfield images (right). Insets show fluorescence of primary and lateral root tips. Scale bar, 1 cm.

In Figure 2e, replace it with the images of luminescence present on the whole plate from the side-by-side plate (shown in Extended Data Fig. 2c), because Fig. 2e doesn't really show what's described in the text (Lines 160-163). The text describing Extended Data Fig. 2c (Lines 165-167) should then refer to Fig. 2e.

We replaced panel 2e with the side-by-side from Extended Data Fig. 2c and updated both the caption and main text to reflect the change. The relevant main text is pasted here for review [lines 162 - 175]:

"The OMY-independent reporter exhibited strong luminescence across the entire plate regardless of plant genotype, while OMY-dependent luciferase activity was only observed near sfGFP-MfnG expressing plants (Fig. 2e, Extended Data Fig. 2a-c). Plants expressing proUBQ10::sfGFP-MfnG produced strong luminescence peaks with the OMY-dependent reporter (~3.2-fold above midpoint intensity) (Fig. 2f-g, Extended Data Fig. 2c). We also observed small luminescence peaks with the OMY-independent reporter at each seedling (~1.2-fold above midpoint intensity), suggesting bacterial density may increase near the roots and contribute to a local increase in luminescence signal (Extended Data Fig. 2a). Wild-type plants co-cultivated with the OMY-dependent strain showed a similar, 1.4-fold increase in luminescence at the root (Fig. 2f-g, Extended Data Fig. 2c). Excitingly, when OMY-producing transgenic plants were grown side-by-side with unmodified wild-type plants, the luminescence signal was concentrated around the transgenic plants (Fig. 2e-g). Thus, plant-produced OMY enables genotype-specific induction of bacterial gene expression.

Line 201: the described results actually are shown in Extended Data Fig. 4f (not Extended Data Fig. 4e).

We swapped panels E & F in Extended Data Fig. 5 to correct this error. We note that the figure numbering was updated during revision to facilitate the incorporation of new data [lines 208-211].

Line 202: "Yet, exogenous OMY treatment of wild-type seeds had minimal effect on germination" – This is 4e. Panels should be flipped in Extended Data Fig. 4.

We swapped panels E & F in Extended Data Fig. 5 to correct this error. We note that the figure numbering was updated during revision to facilitate the incorporation of new data [lines 208-211].

In Figure 4a, fluorescence signal located in root caps is a little difficult to see. Would it be possible to show an overlay of the GFP on the brightfield image?

We updated Fig. 4a to include a brightfield overlay (right) and a clearer zoom in of the primary and lateral root tips to see fluorescence. The figure panel and caption are reproduced here for review [lines 661-666]:

Figure 4. Conditional MfnG expression reduces fitness costs while maintaining plant-based bacterial control. (a) sfGFP expression in homozygous proBRN1::sfGFP-MfnG seedlings 11 days post-stratification. Widefield fluorescence microscopy (left, 500 ms exposure) and composite brightfield images (right). Insets show fluorescence of primary and lateral root tips.

Similarly, in Figure 4b, the image appears very dim (at least in my copy), and it is difficult to see GFP expression throughout the plant.

The fluorescence signal is concentrated in the youngest parts of the root after induction. We increased the brightness of the images to make the expression pattern clearer.

Figure 4. Conditional MfnG expression reduces fitness costs while maintaining plant-based bacterial control. (b) sfGFP expression in T1 hemizygous transformants of proXVE::sfGFP-MfnG seedlings at 0 hours (left) and 24 hours (right) after transfer to media containing 20 μ M α -estradiol. 150 ms exposure in the GFP channel.

In Extended Data Fig 2d (described in Lines 228-232), luminescence in the central panel is very difficult to see. Interestingly, it's easier to see in the composite image.

We updated Extended Data Fig. 2 to change the luminescence scale bar. This makes the signal a little easier to see in all OMY-dependent co-cultivation images (Extended Data Fig. 2d). Since the proBRN1 results are admittedly still dim, we quantified luminescence across the plates to make it easier to compare luminescence signals across conditions [Methods lines 1224 – 1252]. A zoom in on the proBRN1::sfGFP-MfnG panel from Extended Data Fig 2c are reproduced below for review:

In Extended Data Fig. 5e-g, it's not clear that the purple bars (or histograms) represent co-cultivation of UD1022 with wild-type Arabidopsis plants. Perhaps add a fourth row on the x-axis in (f) and (g) to indicate wt plants?

We have updated the x-axis in this figure panel to indicate wild type plants. The new Extended

Data Fig. 6cd is pasted below for review. We note that the figure and panel numbering was updated during revision to facilitate the incorporation of new data.

Extended Data Fig. 6. OMY-dependent gene expression in *Bacillus subtilis* UD1022 in liquid media and soil.

In Fig. 5c (and Extended Data Fig.6), why is the luminescence brightest at the root tips if MfnG is ubiquitously expressed? Discuss the reason(s) for this observation.

We believe three factors likely contribute to the brighter luminescence observed at proUBQ10::sfGFP-MfnG root tips co-cultivated with the OMY-dependent UD1022 reporter strain. First, root tips contain a high density of small, actively dividing cells, which leads to higher gene expression per cm root length in the root tip compared to more mature root tissue. Second, root tips are the primary site of root exudation. Thus, they likely secrete more OMY than other root regions, increasing local OMY availability for bacterial signaling. Third, UD1022 may be able to utilize root-secreted nutrients more effectively than PY79 in this location, potentially increasing bacterial density near the tip and further contributing to the observed luminescence signal. We added text to the results section to directly address the difference between PY79 and UD1022 signal [lines 291 - 294]:

“UD1022 displayed luminescence patterns similar to PY79, with signal brightest at the root tips despite ubiquitous MfnG expression (Fig. 5f, Extended Data Fig. 7). The root tip signal was more pronounced with UD1022, which may reflect its greater ability to utilize root exudates for growth.”

In line 289, this should be Extended Data Fig. 7.

This figure reference has been corrected and we note that figure numbering was updated during revision [lines 304-306]:

“The resulting composite plants contain wild-type shoots and a mixture of transgenic hairy roots and non-transgenic roots emerging from the shoot44 (Extended Data Fig. 8).”

Figure 1 legend: describe other genetic components, pVeg, pSer, etc., shown in panel b.

The figure legend was updated to include information on the other genetic components. The legend for Fig. 1b now reads (changes underlined) [lines 588-596]:

“(b) Genetic code expansion components introduced to *B. subtilis* to enable OMY-dependent gene expression. An engineered variant of the *Methanocaldococcus jannaschii* tyrosyl-tRNA synthetase (MjTyrRS)26 and orthogonal suppressor tRNA (tRNA^{Tyr}CUA)26, were constitutively expressed by the pVeg73 and pSer27 promoters, respectively. The Gene Of Interest, GOI, was engineered to have an in-frame amber stop codon, TAG, for site-specific ncAA incorporation and was expressed constitutively by a pHyperspank74. A chloramphenicol resistance cassette, CmR75, and kanamycin resistance cassette, KanR76 were used for selection of each construct upon genome integration and both were flanked by LoxP77 sites for subsequent excision by Cre recombinases (Methods).”

Extended Data Fig. 5 legend, Line 635: 1 day is shown in panel b, and 3 days is shown in panel c.

The figure legend has been revised to [line 701]

“Cells were extracted from the soil 1 day (c) or 3 days (d) after inoculation.”

Minor typos:

Line 264: delete “next”

We deleted the second “next” to correct this error. The sentence is now [line 281]:

“Next, we tested whether plant-produced OMY could activate the UD1022 reporter in soil.”

Figure 6 legend, “*R. rhizogenes* transfers its plasmids into tomato cells,...”, it should say T-DNA, not plasmids.

The figure legend has been revised to [lines 714-716]:

“*R. rhizogenes* transfers its T-DNA into tomato cells, which induces hairy root growth and produces composite plants with wild-type shoots and a mixture of transgenic (magenta) and non-transgenic (grey) roots.”

Line 676, in Methods: rifampicin concentration is missing.

The concentration has been added [line 844]:

“rifampicin 15 µg/ml (GoldBio R-120)”

Reviewer #3 (Remarks to the Author):

Zhong et al. demonstrated that non-standard amino acids can be used as a platform for plant-based control of microbial activity. Using genetic code expansion, they engineered O-methyl-L-tyrosine (OMY)-dependent control of protein expression in *Bacillus subtilis*. They then stably incorporated OMY biosynthesis into *Arabidopsis thaliana* and evaluated OMY phytotoxicity. Given the detrimental impacts of constitutive OMY biosynthesis on *A. thaliana*, the authors

utilized tissue-specific and conditional production of OMY to reduce fitness costs while maintaining plant-based bacterial control. The authors also tested OMY-based bacterial control using composite tomato and poplar plants to demonstrate that their system can be extended to other plant species. While the overall study is useful and expands the current repertoire of signaling molecules for modulating plant-bacterial interactions, the results are mainly descriptive and qualitative. Additional quantification beyond visual luminescence intensity scales and better spatial resolution would be helpful to understand how effective OMY-based control is in co-cultivation systems.

We thank the reviewer for their careful analysis of our manuscript and their helpful suggestions. We agree that additional quantification is useful for understanding our system. To quantify luminescence with spatial resolution, we created luminescence intensity profiles across the x-axes of our co-cultivation plates. We used these profiles to measure fold change in luminescence at each root vs the midpoint between plants and to calculate maximum luminescence intensity for each sample. These measurements proved useful for quantifying luciferase induction during co-cultivation and better understanding the drivers of the luminescence spatial pattern. Specific findings related to the quantification and methodological details are provided below.

The value of this system compared to other molecules such as rhizopines, sucrose, and opines appears to be the potential orthogonality of non-standard amino acids for targeted plant-bacteria communication. However, this was not clearly demonstrated in relevant conditions including the use of non-sterile soils or in the presence of multiple bacterial species to understand off-target effects.

We agree that demonstrating orthogonality in more ecologically realistic conditions, including non-sterile soils and multi-species microbial communities, is an important next step for evaluating this system's potential advantages over previously described plant-bacteria signaling molecules. We are actively developing experiments, in collaboration with a microbial ecologist, to test the effects of OMY on native soil microbial communities and system robustness using field-collected soil. We believe this is an important and substantial undertaking that merits its own dedicated study, and we have added a statement to the Discussion [lines 340 - 343] noting this as an important direction for future work:
"Testing this system in non-sterile soils with diverse native microbial communities will also be important for evaluating potential off-target effects and confirming that OMY-based signaling remains specific to engineered strains in complex, multi-species environments."

The major emphasis of the paper appears to be engineering OMY biosynthesis in plants and controlling its phytotoxicity. Much more supporting evidence and additional mechanistic validation would be needed to strengthen the authors' claims. Therefore, I do not think that this manuscript is acceptable for publication in Nature Biotechnology. My other questions are listed below.

We welcome the opportunity to strengthen the manuscript, and we have focused our efforts on the reviewer's specific questions below.

1. The authors investigated the effects of OMY on phytotoxicity, but what about the effects of OMY on bacteria? Is there a concentration dependent effect that could impact bacterial fitness? Forti et al. (2025) report that OMY can be consumed by other bacteria while Zer et al. (2025) report that m-Tyr, another non-proteogenic amino acid, is highly toxic to various cyanobacteria. We performed an additional experiment to determine the impact of OMY on the growth of *B. subtilis* and observed no difference in doubling time up to the maximum tested OMY concentration (1 mM). Since compound toxicity can vary between bacterial species, as illustrated by the reviewer's example of m-Tyr toxicity in cyanobacteria (Zer et al., 2025), future work could test OMY's effects on additional bacterial species, particularly those relevant to target deployment environments. The *B. subtilis* growth curves and doubling times were included in Extended Data Fig 4 [lines 769-777] and addressed it in the main text [lines 184 - 185]:

"Addition of OMY to bacterial growth media did not affect bacterial growth rate (Extended Data Fig. 4a,b)."

Extended Data Fig. 4. *B. subtilis* growth and protein expression with OMY. (a) Growth of PY79 expressing the amino-acyl tRNA synthetase and tRNA for ncAA incorporation in 1/2x MS media supplemented with 1.5% sucrose, 10 mM L-glutamate, and varying concentrations of OMY. Each plot contains the growth for three individual biological replicates. The OMY concentration is indicated in the lower right corner of each plot. (b) Growth rates calculated for

curves in (a). Data shows mean and standard deviation across three biological replicates. Statistical significance was determined using one-way ANOVA and Dunn's test against the no OMY control ($\alpha = 0.05$).

2. The authors report OMY biosynthesis in the μM range (3.1 μM from 18 ubiquitously expressing seedlings and 0.73 μM from 50 root cap-specific seedlings) after 48 hrs. In their bacterial reporter assays, they show significant fold-change in induction, but at 100 μM OMY. This raises the question of what is the actual fold-change in induction at plant-produced OMY conditions? Is the level of OMY production from plants meaningful? (See point 3) Reviewers 1 & 2 had similar questions. To address these comments, we performed additional reporter activation experiments at lower concentrations of OMY. We found statistically significant activation with as little as 0.05 μM (1.07-fold) and 0.5 (7.7-fold) μM OMY for PY79. We added this data as Extended Data Fig. 4c-d [lines 769, 777-781].
Extended Data Fig. 4. *B. subtilis* growth and protein expression with OMY. (c-d) Fluorescence of *B. subtilis* PY79 (c) or UD1022 (d) expressing an OMY-dependent mNeonGreen reporter across a range of OMY concentrations in minimal media. Fluorescence measured via flow cytometry. Statistical significance was evaluated using a one way ANOVA with Tukey HSD posthoc test (** $p < 0.01$, **** $p < 0.0001$).

Extended Data Fig. 4c reference [lines 237 - 242]:

"Because this concentration falls below the initially characterized range (Fig. 1c), we tested incorporation at lower OMY concentrations. Using flow cytometry to sensitively measure OMY-dependent mNeonGreen fluorescence, we found induction at OMY concentrations as low as 0.05 μM (1.67-fold) and 0.5 μM (7.7-fold) (Extended Data Fig. 4c). Thus, the reporter remains sensitive to OMY below the 1 μM tested with plate reader fluorescence."

To address the question about fold change, we quantified the luciferase signal in our co-cultivation experiments by measuring the luminescence intensity profile across the x-axis of each plate (Methods lines 1224 - 1252). We found that the plants ubiquitously or root cap-specifically expressing sfGFP-MfnG were able to generate ~3.2-fold and ~2.2-fold increase in luciferase signal at the root relative to the midpoint between plants, respectively. Comparing maximum luminescence signals showed a 2- (root cap specific) to 15-fold (ubiquitous) increase in reporter gene expression when the OMY-dependent bacteria were co-cultivated with transgenic vs wild type plants. Whether these fold changes in bacterial gene expression are meaningful to the plant will depend on what the bacteria is producing in an OMY-dependent manner. Phytohormones, which are potent growth regulators, would likely be effective at low concentrations.

We note that quantification showed a small increase in luciferase signal for OMY-dependent bacteria co-cultivated with wild type plants (~1.4-fold) and OMY-independent bacteria co-cultivated with all plant genotypes (~1.2-fold). We suspect that this signal is due to a combination of higher bacterial density at the root and, in the OMY-dependent bacteria, low-level readthrough of the amber stop codons. Despite this background signal, plant-produced OMY was sufficient to drive substantially higher, genotype-specific bacterial induction.

All of the text and figure changes are highlighted in the revised manuscript. Luminescence quantification was added to Fig. 2, Fig. 4, and Extended Data Fig. 2. The Results [lines 165 – 172, 237 – 244, 250 – 255] were updated to discuss these analyses. A representative subset of the figures that now contain quantified luminescence are pasted below for review:

Panels g-j from Fig. 4 show an example of luminescence quantification [lines 674-680]:

Figure 4. Conditional MfnG expression reduces fitness costs while maintaining plant-based bacterial control. (g) Co-cultivation of OMY-dependent *B. subtilis* PY79 luciferase reporter strain (as in Fig. 2e) with proBRN1::sfGFP-MfnG seedlings. Scale bars, 1 cm. (h) Luminescence intensity profile across the x-axis of the proBRN1::sfGFP-MfnG plate in (g) (orange) and wild type Col-0 plate in Extended Data Fig. 2c (grey). (i,j) Fold change (i) and maximum (j) luminescence intensity for wild-type Col-0 (grey, $n=5$) and proBRN1::sfGFP-MfnG (orange, $n=5$) samples in (h). Statistical significance was evaluated using a Welch's test (** $p < 0.01$).

For comparison, here is the plate image showing luminescence signal from wild type Col-0 plants co-cultivated with OMY-dependent bacteria using the new luminescence scale (10,000 signal threshold). This is a panel from Extended Data Fig 2c:

Results section referencing Extended Data Figure 4 and the new Figure 4 panels [lines 233 - 246] (changes underlined):

“We next tested whether conditional expression produces sufficient OMY for bacterial control. LC-MS analysis of conditioned media detected OMY in proBRN1::sfGFP-MfnG samples, though at lower concentrations than proUBQ10::sfGFP-MfnG (0.73 μ M from 50 root cap-specific seedlings versus 3.1 μ M from 18 ubiquitously expressing seedlings cultured in 12 mL media), which is consistent with BRN1 expression being restricted to ~19% of root cells^{37,39}. Because this concentration falls below the initially characterized range (Fig. 1c), we tested incorporation at lower OMY concentrations. Using flow cytometry to sensitively measure OMY-dependent mNeonGreen fluorescence, we found induction at OMY concentrations as low as 0.05 μ M (1.07-fold) and 0.5 μ M (7.7-fold) (Extended Data Fig. 4c). Thus, the reporter remains sensitive to OMY below the 1 μ M tested with plate reader fluorescence. In co-cultivation assays, proBRN1::sfGFP-MfnG plants still activated OMY-dependent NanoLuc expression ~2.2-fold above midpoint intensity (Fig. 4g-j, Extended Data Fig. 2c). Though luminescence was lower than observed with proUBQ10::sfGFP-MfnG plants, peaks at individual plant locations remained well defined.”

We added Supplementary Figure 3 to show readers the peak (red arrow) and midpoint locations (green dashed line) used to calculate the fold change and max intensity luminescence values shown in Fig. 2, Fig. 4, and Extended Data Fig. 2. Here is an example panel from Supplementary Figure 3:

We added a new Methods [lines 1224 – 1252] section to describe the quantification method:

“Quantification and analysis of luminescence intensity

Raw luminescence (ChemiBlot channel) images (16-bit) were used for quantification with Fiji. Square ROIs were drawn on the corresponding brightfield image (Membrane channel) following grid borders on the petri dish. ROIs were propagated to the luminescence image and the Plot Profile tool was used to plot the vertically averaged pixel intensity at distances along the x-axis, starting with 0 cm at the left extreme.

Further data analysis was implemented in Python 3 using numpy, scipy (v1.11), and pandas. Local maxima (peaks) in the luminescence intensity profile corresponding to the location of individual seedlings were extracted as follows. Each profile was smoothed with a uniform (boxcar) moving-average filter (kernel width = 1/10 of the expected inter-peak spacing) to reduce noise. Candidate peaks were identified using scipy.signal.find_peaks with a minimum inter-peak distance set to half the expected spacing. The prominence threshold parameter was iteratively lowered from 30% to 1% of the intensity value range until at least n (the number of seedlings) peaks were found. Only the n most prominent peaks were retained. If fewer than n peaks were detected, the largest inter-peak gap was bisected and the local maximum within that gap was added. Each detected peak was then snapped to the local maximum of the unsmoothed profile within ± 3 data points (~0.08 cm). For PY79 negative-control plates, since no peaks were expected, peak positions were assigned to fixed equidistant intervals along the x-axis.

For each peak at position x_0 , two midpoints were defined: x_1 halfway between x_0 and the adjacent peak to the left (or the profile start), and x_2 halfway to the adjacent peak to the right (or the profile end). The peak (maximum) intensity y was taken as the exact (unsmoothed) value at x_0 . The baseline intensities y_1 and y_2 were computed as the mean value within ± 0.1 cm of x_1 and x_2 , respectively, and averaged to yield the base intensity $y_0 = (y_1 + y_2)/2$. The normalized luminescence ratio (fold change) was calculated as y/y_0 . Peak and midpoint locations are annotated in Supplementary Fig. 2. Claude Science Opus 4.6 was used in the development of the Python analysis script.”

3. The data presented are endpoint measurements taken after often 4 days of co-cultivation. Given the low concentration of OMY biosynthesized per plant and the amount of OMY needed to produce fold-changes in luminescence, reporting of earlier timepoints and the OMY concentration over time would provide a clearer picture of if this signaling process is dynamic and can be controlled. Can you achieve a range of bacterial protein production or is it just off and on with this system once a minimum threshold of OMY is reached?

We expect this process to be dynamic. This is supported by two lines of evidence in our data. First, our flow cytometry titration experiments show that reporter induction increases progressively with OMY concentration, from no significant induction at 0.005-0.05 μ M to 7.9-fold at 0.5 μ M, 48.9-fold at 5 μ M, and 111-fold at 50 μ M (Extended Data Fig. 5c-d), demonstrating that this system produces a graded, dose-dependent output rather than a simple on/off switch. Second, in co-cultivation experiments, the range of maximum luminescence intensities observed across different plant lines and conditions (~88 au with wild-type, ~187 au with proBRN1::sfGFP-MfnG, ~1300 au with proUBQ10::sfGFP-MfnG, and ~2000 au proXVE::sfGFP-MfnG with or without β -estradiol) further suggests that bacterial output scales with the amount of

OMY available, rather than switching on uniformly once a minimum threshold is reached.

4. The authors used sterile soil throughout their experiments, but it would be informative to understand how orthogonal this technology is in non-sterile soils and in the presence of other soil microbes that could consume the OMY, reducing the local concentration, or have other unintended effects.

We agree that testing this system in non-sterile soils, in the presence of diverse native microbial communities, is an important next step for evaluating its orthogonality and potential off-target effects, including OMY consumption by other soil microbes. As noted in our response above, we are actively developing experiments, in collaboration with a microbial ecologist, to test incorporation and the effects of OMY on native soil microbial communities using field-collected soil. We have added this as an important direction for future work in the revised Discussion [lines 340 - 343]:

“Testing this system in non-sterile soils with diverse native microbial communities will also be important for evaluating potential off-target effects and confirming that OMY-based signaling remains specific to engineered strains in complex, multi-species environments.”

5. It would be interesting to understand OMY distribution surrounding the plants and how that corresponds to the observed luminescence intensity and bacterial density. The claim that plant-produced OMY enables genotype-specific induction of bacterial gene expression would be strengthened if they labeled the bacteria to decouple potential localization effects from luminescence. Is stronger signal due to more bacteria being present around those plants? Is there a concentration gradient of OMY from the plant outwards that corresponds to luminescence profiles?

We expect that the plants produce an OMY concentration gradient where OMY is highest at the root and diffuses through the agar matrix becoming less concentrated as a function of distance from the root. We also expect more bacteria near the root where exudates can provide nutrients to the bacteria. Though we did not directly measure OMY concentration or bacterial density during co-cultivation, our luminescence quantification helps assess the relative contribution of these two factors to the observed luminescence profile. The OMY-independent reporter showed small luminescence intensity peaks at every plant regardless of genotype (~1.2-fold), suggesting that this signal reflects bacterial density rather than OMY. The OMY-dependent reporter showed a similarly small fold change on wild-type plants, which do not produce OMY, but a substantially larger fold change (2.2- to 3.2-fold) on OMY-producing plants. Together, these results suggest OMY concentration, not bacterial density, is the dominant driver of genotype-specific induction. We added a discussion of these results and potential approaches for measuring OMY or bacteria during co-cultivation to the Discussion [lines 345 - 355]:

“Interestingly, bulk measurements of OMY did not reflect the level of reporter activation at plant roots in co-cultivation experiments. This is likely because bulk measurements underestimate OMY concentration at the root, where bacteria contact the plant directly. Though OMY concentration gradients may explain the strong luminescence of OMY-dependent reporter strains grown with MfnG-expressing plants, the OMY-independent reporter also showed a modest increase in luminescence near the roots of every plant genotype, suggesting bacterial cell density may increase near the roots and contribute to a local boost in luminescence signal. Though we did not directly measure OMY concentration or bacterial cell density at the root, labeling bacteria with a constitutive fluorescent marker, quantifying OMY extracted from the gel matrix, or counting bacterial CFU at different plate locations would help measure the contribution of chemical and cell concentration gradients to reporter signals in future work.”

6. In Figures 1 and 5, the authors switch to reporting data in mM despite using uM for every other graph. It would be beneficial to stay consistent with the units to understand the magnitude of the dose.

Figures 1d, 5b and the corresponding legends have been updated to μ M [lines 600-601; 695].

7. Figure 4, unclear what g-h caption “event #4, event #1, right three, event #2, left three” means.

Stable plant transformation randomly inserts transgenes into the plant's genome. Each independent insertion is called a transformation “event,” and different events can vary in expression level or phenotype due to differences in insertion site (even with the same transgene construct). Plant biologists typically screen multiple independent events for a given construct and select one or more representative lines for further study. Event numbers (e.g., event #4, event #1, event #2) refer to these independent lines and will help the reader keep track of which plants were used for each of our experiments. In Fig. 4k, “right three” and “left three” indicate the positions of three seedlings each from events #1 and #2 on the same plate. We have clarified this in the Methods [lines 1059-1061]:

“For each construct, we screened multiple independent T1 events and selected one or more representative lines, denoted by event number, for further characterization.”

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed my substantive concerns, and the manuscript is considerably improved. Before acceptance, I recommend correcting the following minor points:

The rebuttal reports 1.07-fold induction at 0.05 μ M OMY in one place, whereas the manuscript and subsequent rebuttal text report 1.67-fold; please verify and correct this discrepancy.
At lines 50–51, defining ncAAs as “a class of non-proteinogenic amino acids” is conceptually imprecise. Consider: “Non-canonical amino acids (ncAAs), amino acids outside the canonical translational repertoire...”
The Figure 5 title still describes UD1022 as a “plant growth-promoting bacterium.” For consistency with the qualified wording in the text, consider “OMY-dependent gene expression in the plant-associated bacterium *Bacillus subtilis* UD1022.”
At line 339, replace “in each bacteria” with “in each bacterial strain.”

These are editorial corrections only; no further experiments are required.

Reviewer #2

(Remarks to the Author)

The authors have properly addressed my previous concerns and suggestions to improve the manuscript, particularly with improvements to fluorescence and luminescence images shown. I appreciate the thorough point-by-point response letter showing not only the authors' responses, but also the corresponding modifications made to the manuscript and extended data.

New experiments addressed concerns (shared by all reviewers) regarding the toxicity of OMY over-production to plants and showed reporter gene activation in bacteria with much lower concentrations of OMY, which also addresses, at least in part, some concerns with real-world applications of this technology. The manuscript has been significantly improved, and I have no further concerns or suggestions.

Reviewer #3

(Remarks to the Author)

The authors did a good job of incorporating the reviewer comments into the manuscript. The additional information regarding their methodology, quantification, limitations of the work, and future efforts to address open questions was very appreciated.

The manuscript is improved, but there are still a few comments that should be addressed prior to publication.

1. Extension of the dose-response curve to lower OMY concentrations is important and I'm glad they added this data since it was brought up by all three reviewers, suggesting that many readers would have similar thoughts. In Extended Data Fig 4d, the fluorescence at 0.050 μ M is shown as ns. Is that correct? It seems a bit surprising since visual comparison of the data for 0-0.050 μ M between 4d and 4c makes it seem like UD1022 has a larger increase in fluorescence between 0 and 0.050 μ M than what is observed in 4c (where fluorescence at 0.050 μ M is significant). It is difficult to know though without higher resolution in the y-axis values.
2. The authors corrected the mM to μ M in the text legend for 1d, but not in the graph legend itself where it still reads as 0.1 mM.
3. In 1a, the figure still has nsAA instead of ncAA
4. Although the authors show no significant effect of OMY concentration on bacterial doubling time for PY79, I am curious if the authors could comment on why there appears to be such significant effects on bacterial growth after around 10 hrs, especially as the OMY concentration increases from 10-1000 μ M.
5. The location of where Extended Data Fig 4a,b are referenced could be better placed in the text. Where it is now seems a bit abrupt since that whole section is focused on plant toxicity and it is just added as a sentence to the end of the paragraph. Perhaps including it somewhere in the paragraph starting in 233 where they mention Extended Data Fig 4c,d could be better for flow.
6. It would be good to repeat the OMY toxicity experiment for UD1022 as well since the authors are emphasizing how they can parallel their system in a wild isolate and there are some slight differences in performance between PY79 and UD1022.
7. In the “OMY production to crop species” section of the work, the authors are illustrating how their efforts can be extended to more agriculturally relevant crop species. It seems like it would make more sense for the authors to do these experiments with UD1022 rather than PY79 and to report the UD1022 data as the main text figures.

Author Rebuttal letter:

Blue denotes response to reviewers; Red denotes manuscript changes

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors have addressed my substantive concerns, and the manuscript is considerably improved. Before acceptance, I recommend correcting the following minor points:

The rebuttal reports 1.07-fold induction at 0.05 μM OMY in one place, whereas the manuscript and subsequent rebuttal text report 1.67-fold; please verify and correct this discrepancy. The 1.07-fold change was a typo. Fold change in fluorescence upon addition of 0.05 μM OMY is 1.67-fold. The correct value is listed in the manuscript.

At lines 50–51, defining ncAAs as “a class of non-proteinogenic amino acids” is conceptually imprecise. Consider: “Non-canonical amino acids (ncAAs), amino acids outside the canonical translational repertoire...”

This line has been revised as suggested. It now reads “Non-canonical amino acids (ncAAs), which are amino acids outside the canonical translational repertoire, have potential for expanding the toolbox.”

The Figure 5 title still describes UD1022 as a “plant growth-promoting bacterium.” For consistency with the qualified wording in the text, consider “OMY-dependent gene expression in the plant-associated bacterium *Bacillus subtilis* UD1022.”

This figure title has been revised as suggested. It now reads “Figure 5. OMY-dependent gene expression in the plant-associated bacterium *Bacillus subtilis* UD1022”

At line 339, replace “in each bacteria” with “in each bacterial strain.”

This line has been revised as suggested. It now reads “Thus, field deployment may require optimization of the orthogonal translation system and the amber codon number in each bacterial strain with plant hosts in the target environment.”

These are editorial corrections only; no further experiments are required.

Reviewer #2 (Remarks to the Author):

The authors have properly addressed my previous concerns and suggestions to improve the manuscript, particularly with improvements to fluorescence and luminescence images shown. I appreciate the thorough point-by-point response letter showing not only the authors' responses, but also the corresponding modifications made to the manuscript and extended data.

New experiments addressed concerns (shared by all reviewers) regarding the toxicity of OMY over-production to plants and showed reporter gene activation in bacteria with much lower concentrations of OMY, which also addresses, at least in part, some concerns with real-world applications of this technology. The manuscript has been significantly improved, and I have no further concerns or suggestions.

Fantastic, thank you!

Reviewer #3 (Remarks to the Author):

The authors did a good job of incorporating the reviewer comments into the manuscript. The additional information regarding their methodology, quantification, limitations of the work, and future efforts to address open questions was very appreciated.

The manuscript is improved, but there are still a few comments that should be addressed prior to publication.

1. Extension of the dose-response curve to lower OMY concentrations is important and I'm glad they added this data since it was brought up by all three reviewers, suggesting that many readers would have similar thoughts. In Extended Data Fig 4d, the fluorescence at 0.050 μM is shown as ns. Is that correct? It seems a bit surprising since visual comparison of the data for 0–0.050 μM between 4d and 4c makes it seem like UD1022 has a larger increase in fluorescence between 0 and 0.050 μM than what is observed in 4c (where fluorescence at 0.050 μM is significant). It is difficult to know though without higher resolution in the y-axis values.

The reviewer is correct that the absolute increase in fluorescence between 0 and 0.050 μM OMY is comparable between the two strains (PY79: 1.67-fold increase; UD1022: 1.98-fold increase). The discrepancy in statistical significance arises because Tukey's HSD test uses a pooled estimate of variance across all six concentrations tested to calculate the standard error for each pairwise comparison. For UD1022, replicate variance at the two highest OMY concentrations (5 and 50 μM) is substantially larger than for PY79 at the same concentrations, which inflates the pooled variance term and reduces statistical power across all comparisons for that strain, including at 0.050 μM . We confirmed that an unpaired t-test comparing 0 and 0.050 μM alone for UD1022 does reach significance ($p = 0.00002$), supporting the reviewer's

observation that induction is occurring at this concentration. However, the appropriate multiple-comparisons correction reduces power to detect this induction against the full dataset.

2. The authors corrected the mM to uM in the text legend for 1d, but not in the graph legend itself where it still reads as 0.1 mM.

The graph legend has been updated to 100 uM.

3. In 1a, the figure still has nsAA instead of ncAA

The figure text has been updated to "ncAA".

4. Although the authors show no significant effect of OMY concentration on bacterial doubling time for PY79, I am curious if the authors could comment on why there appears to be such significant effects on bacterial growth after around 10 hrs, especially as the OMY concentration increases from 10-1000 uM.

The differences in growth observed after 10h are a common artifact of growing *B. subtilis* in a plate reader beyond the initial stationary phase. During stationary phase, *B. subtilis* can enter many different cell states and this results in heterogeneous growth. As the reviewer noted, we observe heterogeneity at high OMY concentrations in the PY79 data and at low OMY concentrations in the UD1022 data, which is consistent with this being an intrinsic property of *B. subtilis* in stationary-phase and not a consequence of OMY supplementation.

5. The location of where Extended Data Fig 4a,b are referenced could be better placed in the text. Where it is now seems a bit abrupt since that whole section is focused on plant toxicity and it is just added as a sentence to the end of the paragraph. Perhaps including it somewhere in the paragraph starting in 233 where they mention Extended Data Fig 4c,d could be better for flow.

We agree that this transition is abrupt, but we prefer the current placement because it appears in the section about OMY toxicity.

6. It would be good to repeat the OMY toxicity experiment for UD1022 as well since the authors are emphasizing how they can parallel their system in a wild isolate and there are some slight differences in performance between PY79 and UD1022.

We agree that testing OMY toxicity with UD1022 is important for demonstrating extensibility of the system. We conducted the experiment and included the results in Extended Data Fig 4e-f. As with *B. subtilis* PY79, we do not observe toxicity with UD1022.

Lines [294-295]: "Similar to PY79, supplementation of OMY to growth media did not impact the growth rate of UD1022 (Extended Data Fig. 4e, f)."

Extended Data Fig 4e,f caption: "(e) Growth of UD1022 in 1/2x MS media supplemented with 1.5% sucrose, 10 mM L-glutamate, and varying concentrations of OMY at 30°C with three biological replicates. (f) The calculated growth rates for each curve in (e). Statistical significance was determined using one-way ANOVA and Dunn's test against the no OMY control ($\alpha = 0.05$)."

7. In the "OMY production to crop species" section of the work, the authors are illustrating how their efforts can be extended to more agriculturally relevant crop species. It seems like it would make more sense for the authors to do these experiments with UD1022 rather than PY79 and to report the UD1022 data as the main text figures.

We appreciate this suggestion. Based on our co-culture experiments with UD1022 and *Arabidopsis*, which showed strong plant-dependent luciferase expression, we expect that UD1022 would perform comparably to PY79 when cultured with poplar and tomato. However, repeating the crop species experiments with UD1022 would require several additional months of plant growth and data collection. We therefore retained PY79 for the crop species experiments presented here and note that extending this work to UD1022 would be a valuable direction for future studies.

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