Nitrous oxide remediation through transformation of *Oryza sativa* with the *nosZ* gene

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The Earth's temperature has risen by approximately 2.12°F since the 1880s. A large portion of this change has been attributed to increased greenhouse gas (GHG) emissions caused by the spread of industrial and agricultural practices. Nitrous oxide (N_2O) makes up approximately 7% of the United States' total GHG emissions. Although this is a relatively small percentage, N2O is three hundred times more powerful than carbon dioxide as a greenhouse gas, and it stays in the atmosphere for an average of 114 years before naturally breaking down. These characteristics make it a dangerous pollutant that significantly contributes to global warming. Around 70% of anthropogenic N_2O emissions come from farming practices, primarily due to the use of nitrogen-based chemical fertilizers. These products typically contain nitrate and ammonia, to provide plants with the proper amount of fixed nitrogen essential to their survival and reproduction. Excess nitrate in the soil is naturally denitrified by *Agrobacterium* species, releasing N_2O and contributing to GHG emissions. The enzyme nitrous oxide reductase, found in the bacterium *Pseudomonas stutzeri* and encoded by the *nosZ* gene, further metabolizes N_2O into harmless nitrogen gas. Using a disarmed T-binary plasmid from *Agrobacterium tumefaciens*, this project will utilize *Agrobacterium*-mediated transformation to insert a *nosZ* gene construct into the genome of the plant *Oryza sativa* (Asian rice). This genetic construct will allow nitrous oxide reductase to be expressed in the roots of *O. sativa*, and if successful, would lead the plant to break down N_2O and convert it into nitrogen gas, mitigating the exponentially rising atmospheric concentration of N_2O.

Keywords: Nitrous oxide, nitrous oxide reductase, Oryza sativa, Agrobacterium, Agrobacterium-mediated transformation

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Watch a video introduction by the authors at https://youtu.be/aIHbcZrBZPw

Background

Nitrous oxide (N_2O) is a harmful greenhouse gas that persists within the atmosphere for an average of 114 years before decomposition through chemical reactions or photolysis (*Greenhouse Gas Sources*, n.d.; United States Environmental Protection Agency [USEPA], 2022). Human activities such as fertilizer usage, fossil fuel combustion, vehicle usage, nitric and adipic acid production, and waste management account for around 40% of N_2O emissions (U.S. Energy Information Administration [USEIA], 2011; USEPA, 2022). The remainder arise from bacterial processes such as nitrification and denitrification, which involve oxidation of ammonia (NH_3) and reduction of nitrate (Dangal et al., 2019). As a GHG, N_2O traps light and heat energy within the atmosphere, and at higher altitudes in the stratosphere, its atmospheric chemistry contributes to depletion of the ozone layer. These factors make it a significant contributor to global warming (Shankman, 2019).

Total United States GHG emissions were 6,558 million metric tons in 2019, and have continued to increase since then (USEPA, 2022). Although N_2O makes up a relatively small 7% of this total, it strongly absorbs infrared radiation, making it 300 times more potent than carbon dioxide as a GHG, and 10–15 times more potent than methane (Chao, 2012). Because of this potency, N_2O increases the Earth's atmospheric temperature faster than any other GHG (USEPA, 2022). High atmospheric concentrations of N_2O, alongside its ability to hasten warming, therefore pose a global threat that humanity needs to address.

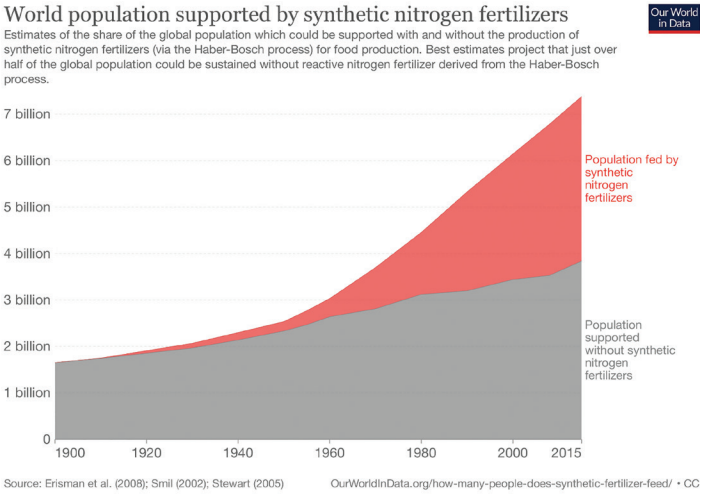

Figure 1. *Increasing rate of global synthetic nitrogen fertilizer reliance in relation to consumption of agricultural goods (Ritchie & Roser, n.d.). As shown by the graph, use of these fertilizers has increased continuously since the 1940s.*

The agricultural industry accounts for 73% of anthropogenic N_2O emissions (USEIA, 2011). This sizable contribution is largely the result of widespread chemical fertilizer usage, which accounts for 87% of total agricultural emissions of N_2O. Chemically produced fertilizers became a part of common agricultural practice in the mid- to late-1940s, as shown in Figure 1 (Hergert et al., 2015). In response to exponential global population growth, many farmers are increasingly relying on methods that improve crop productivity—including greater usage of chemical fertilizers—in order to maximize yields. Recent studies of nitrogen fertilizer effects on productivity have shown that crop yield has increased by 40–60% over the past 25 years in U.S. states such as Kansas, Oklahoma, and Missouri (Mikkelsen, n.d.).

Nitrogen is a critical element in nucleic acids, DNA, RNA, and amino acids, and without it, plant growth and reproduction would be inhibited (Suter at al., 2022). Typically, plants receive nitrogen-containing nutrients through a natural process called nitrogen fixation (Erisman et al., 2015). During this process, soil-resident microorganisms convert atmospheric nitrogen into its fixed forms, NH_3 and nitrate, which plants can easily use as nutrients. Due to the significant demand for crops, microorganisms cannot naturally produce an adequate amount of fixed nitrogen for agricultural plants. To compensate for this, farmers use chemical fertilizers to provide fixed nitrogen to their crops (Sedlacek et al., 2020). In fact, about 90% of NH_3 produced worldwide is used in fertilizer to provide plants with proper amounts of nitrogen. Some of the applied nitrogen compounds in chemical fertilizer are absorbed by the plants' roots, while microbes in the soil digest the rest. Thus, in the presence of excess fixed nitrogen, denitrifying bacteria in the soil will break down nitrate and release N_2O as a byproduct (Sedlacek et al., 2020). As the United States continues to produce more crops for the expanding population, N_2O emission rates are predicted to increase by 24–31% by 2050 (Kanter et al., 2016).

One possible solution to remediate these growing N_2O emissions involves utilizing a multiple copper ion-binding enzyme known as nitrous oxide reductase (N_2OR). Naturally found in *Pseudomonas stutzeri*—a nonfluorescent, denitrifying bacterium—the enzyme converts N_2O into dinitrogen gas (N_2) as the final step of bacterial denitrification (Zhang et al., 2019), a process whereby nitrates present within the soil are converted into free atmospheric N_2 (Encyclopædia Britannica, 2021). The aim of our project is to introduce the *nosZ* gene, which codes for N_2OR, into a plant's genome. We will use *Agrobacterium*-mediated transformation to achieve this, and have selected *Oryza sativa* (Asian rice) as the target plant. As the third most-produced agricultural crop worldwide, *O. sativa* could significantly reduce the N_2O gas concentration if it expressed the N_2OR enzyme (Wallach, 2022). Through the processes of *Agrobacterium*-mediated transformation and floral-dip transformation, we will transfer a T-binary plasmid containing the *nosZ* gene under the control of the *rolD* promoter, a constitutive root-specific plant promoter, into *O. sativa*. In a similar experiment, *Agrobacterium*-mediated transformation was successfully used to generate transgenic tobacco plants expressing N_2OR under control of the same *rolD* promoter (Wan et al., 2011).

Agrobacterium strains typically carry a Ti-plasmid, which is commonly exploited by researchers to introduce new genes into plant genomes. Natural *Agrobacterium* infections of injured plants cause the formation of tumors by transfer of bacterial tumor-inducing DNA (T-DNA) into the plant's genome. The T-DNA that integrates into the host genome is a specific part of the Ti-plasmid (Nester, 2015), and integration also relies on virulence (vir) genes located on the Ti-plasmid, which code for proteins that guide the transformation process. Our design will use a modified two-vector system to transform *O. sativa* with N_2OR. This system utilizes a T-binary vector, along with a modified Ti-plasmid, to accomplish *Agrobacterium*-mediated transformation without inducing tumor growth.

The T-binary vector is a modified plasmid that contains T-DNA repeats and carries the transgene of interest (Kroemer, n.d.). We will insert the *nosZ* gene into the pRI910 T-binary plasmid, then transform the LBA4404 strain of *Agrobacterium tumefaciens* with the resulting construct. The pRI910 plasmid and LBA4404 strain are some of the most commonly used and well-researched tools for plant genome manipulation (Kámán-Tóth et al., 2018). More specifically, the pRI910 plasmid is derived from *Escherichia coli* cloning vectors and contains T-DNA border sequences to promote T-DNA transfer in the desired orientation (Anami et al., 2013; Peralta & Ream, 1985). The T-binary vector will operate in conjunction with a modified T-DNA-less Ti-plasmid containing a vir gene, which will aid in transferring and inserting the gene of interest without causing tumor growth in the plant (Anami et al., 2013). By placing the structural gene coding for N_2OR into the rice plant with a root-specific promoter, we will obtain transgenic plants with the ability to metabolize N_2O into harmless N_2.

Systems level

Our design enables *O. sativa*, one of the most-produced crops globally, to reduce agricultural emissions of N_2O (Figure 2). The chosen system mimics the identical metabolic pathway in a soil bacterium, *P. stutzeri*, that can naturally break down N_2O. As already stated, the system relies on expression of N_2OR, a multicopper enzyme that catalyzes a two-electron reduction of N_2O, forming N_2 and H_2O as its products (Messerschmidt, 2010). To keep the expression of the transgene contained, the root-specific *rolD* promoter will limit the expression of N_2OR to within the plant's roots. In other words, the enzyme should only be present and functional in the roots of *O. sativa*. Other scientists have previously transferred the *nosZ* gene, under control of the *rolD* promoter, into the genome of *Nicotiana tabacum* (Wan et al., 2012). Functioning together, the promoter and enzyme within the tobacco plant were shown to reduce N_2O concentrations in their experiment. To express the N_2OR enzyme within *O. sativa*, we will similarly introduce the *nosZ* gene and *rolD* promoter into *O. sativa* via *Agrobacterium*-mediated transformation. When the transformants interact with N_2O, the N_2OR enzyme will reduce the N_2O, a potent GHG, breaking it down into harmless N_2 and H_2O. We expect that the *nosZ* transgenic plants will continue to grow and produce seeds, and since the gene will be integrated directly into the *O. sativa* genome, the offspring of these plants will possess the *nosZ* gene.

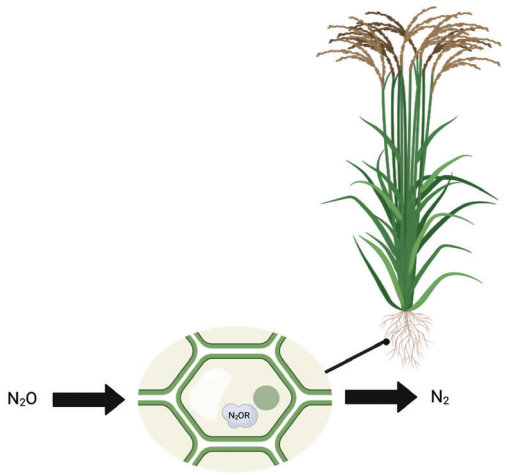

Figure 2. *Nitrous oxide reduction in the roots of O. sativa.*

Device level

Agrobacterium is considered "nature's genetic engineer" due to its ability to naturally transfer genes into plant genomes (Figure 3). Because of this, *Agrobacterium*-

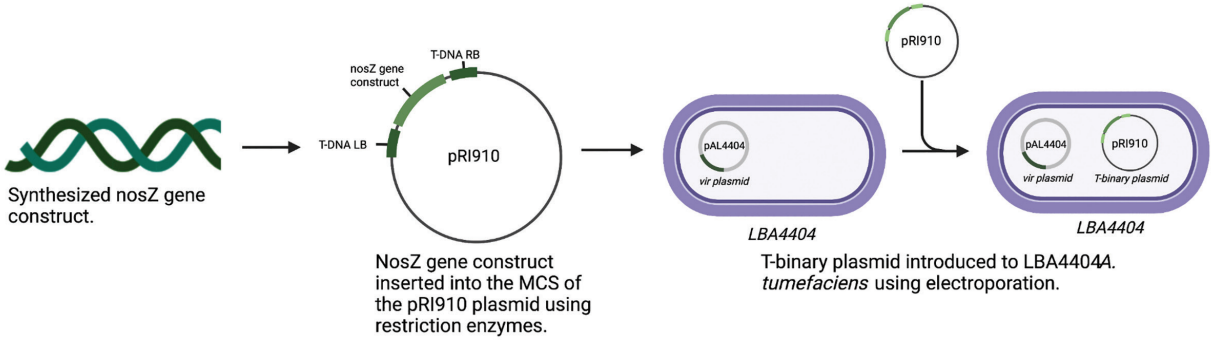

Figure 3. *Transformation of A. tumefaciens strain LBA4404 with the pRI910 plasmid containing the nosZ gene construct.*

mediated transformation is the most commonly used method to genetically modify plants (Harper, 2022). This approach is quicker and cheaper than traditional plant breeding methods due to the ability of agrobacteria to directly integrate genes conferring specific traits through single transformation events, along with their capacity for stable integration within transgenic plants (Kroemer, n.d.; Hwang et al., 2017). For these reasons, *Agrobacterium tumefaciens* strain LBA4404 is an ideal chassis for the genetic modification of *O. sativa*. The LBA4404 strain is easily accessible, is considered a biosafety level 1 (BSL-1) organism, and is a commonly used strain for plant transformation. It has previously been shown to be efficient for transformation of *O. sativa* (*Agrobacterium tumefaciens Electrocompetent Cells*, n.d.; Aldemita & Hodges, 1996) In addition, this strain can be used in conjunction with the pRI910 T-binary vector carrying our gene of interest, as it has been modified to contain the disarmed Ti-helper plasmid pAL4404 (*LBA4404 Electrocompetent Agrobacterium*, n.d.). The pAL4404 plasmid contains a vir gene, which codes for a protein that aids in transferring the gene of interest into a plant, as discussed above (Figure 4). The pRI910 *nosZ* construct will be introduced into LBA4404 using electroporation. If the transformation is successful, the *A. tumefaciens* cells will carry both the modified pRI910 plasmid and the helper pAL4404 plasmid.

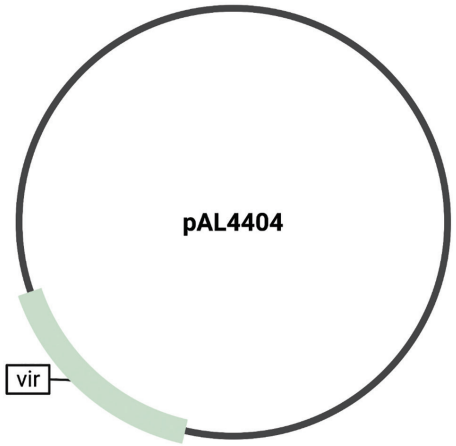

Figure 4. *Helper plasmid pAL4404.*

Parts level

The *nosZ* gene ("Nucleotide," 2022) codes for nitrous oxide reductase, which exists in cells as a homodimer and requires various maturation factors for the protein to be functional (Pomowski et al., 2011; Zhang et al., 2019). A well-studied example of this enzyme is found in *P. stutzeri*, and catalyzes the final step within nitrate reduction (Messerschmidt, 2010). We will insert a genetic construct (Figure 5) containing the *rolD* promoter, synthesized *nosZ* gene, and NOS terminator into the pRI910 T-binary plasmid. As outlined above, the *rolD* promoter was chosen for localized root-specific expression, while the NOS terminator is widely used for plant transformation constructs (Wan, 2011; Xu, 2014). The pRI910 plasmid contains various characteristics suitable for performing stable *Agrobacterium*-mediated transformation, including a T-DNA region that has proven beneficial for the development of transgenic rice plants. Furthermore, the cloning sites in pRI910 are located closer to its right T-DNA border than to its selection marker, *nptII*, a mutant-type kanamycin resistance gene used for plant selection (Figure 6). This location increases the likelihood of stable gene integration as the gene is less likely to be deleted during protein synthesis (*Agrobacterium tumefaciens Electrocompetent Cells*, n.d.). The pRI910 plasmid will be used together with the pAL4404 helper plasmid. This plasmid only contains the T-DNA vir region, and will aid in transferring the gene of interest.

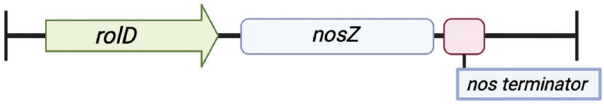

Figure 5. *Genetic construct containing the rolD promoter, nosZ gene, and NOS terminator.*

Safety

When genetically altering crops intended for human consumption, there are many safety concerns regarding the effects of these alterations on the human digestive system. Slight errors or off-target effects in the *Agrobacterium*-mediated transformation may lead to unsafe rice crops for human consumption. For the *Agrobacterium* itself, it is classified as BSL-1 and scientists consider it an organism of low pathogenicity (Hulse et al., 1993). *Agrobacterium tumefaciens* has only shown limited infections within immunocompromised patients. Therefore, agrobacteria are generally considered virtually harmless when appropriately handled in a lab (Petrunia et al., 2008). We will use

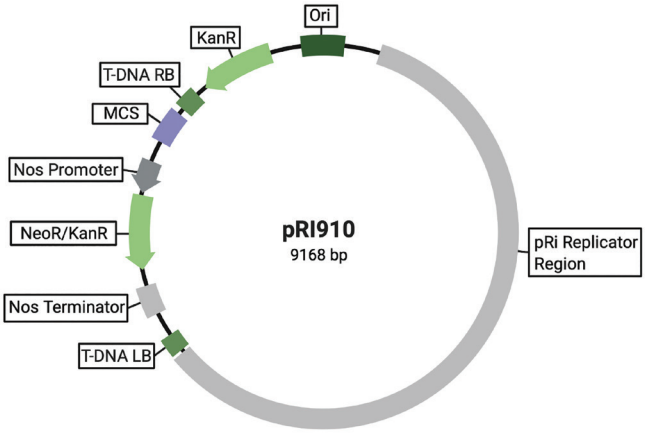

Figure 6. *T-binary plasmid pRI910.*

established methods such as the floral-dip technique for our *Agrobacterium*-mediated transformation, as well as other standard verification methods—including PCR, RT-PCR, Western blot, and the methyl viologen-linked activity assay—to assess the success and safety of our experiments. We will also complete all experiments and procedures in a BSL-1 lab in compliance with all implied regulations. Additionally, we will use equipment that can regulate the development of the altered plants by replicating the growth conditions of standard rice plants. We will test a quantity of juvenile *O. sativa* throughout their development for a comprehensive data set, and all necessary verification of safety and success.

One goal of this project is to increase the natural yield of *O. sativa* without harming the environment surrounding the plant or the human consumer. To ensure success with these specifications, we will evaluate multiple transgenic *O. sativa* plants over an entire life cycle and monitor their health, lifespan, and yield. We will also continue to test nitrogen levels in the soil and air around the plants of interest to determine if our alterations are achieving the intended purpose. Testing other natural compounds commonly found in the plants' environment will allow us to detect any unforeseen imbalances in the present elements needed for *O. sativa* to grow, and the other plants and animals in its natural habitat.

Many genetically modified organisms (GMOs) are under harsh review, and are at the center of continued agricultural debates on whether they are truly safe for consumption (Giraldo et al., 2019; Devos et al., 2016). When working to genetically modify plants—especially crops intended for human consumption—we will consider allergens, toxicity, and horizontal gene transfer (HGT). While our project is still in the hypothetical design process, we are unable to accurately predict the frequency of HGT of our transgenic DNA. Our intention is to incorporate the *nosZ* gene from *P. stutzeri*, a pre-existing bacterium residing in the surrounding soil of the *O. sativa* plant, into the genome of the latter. Current research is still inconclusive on the ecosystem risks associated with HGT of recombinant DNA occurring naturally in the host's environment. Some researchers consider the preexistence of the transgenic DNA to cause skewed data on its spread throughout the environment. Others argue that the risk of HGT is likely much lower if the transgenic DNA has been present in the desired environment without causing disastrous transformations of surrounding organisms (Nielsen et al., 2007; Warwick et al., 2009). In theory, by inserting the *nosZ* gene into *O. sativa*, there will not be any adverse side effects as *P. stutzeri* naturally and safely inhabits this environment, with expression of its *nosZ* gene. More conclusive risks will be determined as this project develops in a more tangible form, and as scientists in the wider field complete further research on the issue. Our team will address the appropriate safety tests, and make edits to our design and procedure in compliance with current and future regulations.

Discussions

If the modified *O. sativa* plant successfully decreases N_2O emissions, the system could be combined with other enzymes to address additional agricultural stressors. Farmers apply approximately 115 million metric tons of nitrogen-based chemical fertilizers annually, at a cost of roughly $887 per ton (Quinn, 2021). However, crops only take up 35% of this total, resulting in 75 million metric tons—or approximately $66.5 billion worth—of nitrate runoff, which contaminates rivers, lakes, and other natural environments. Eventually, nitrogen-breathing microbes in these polluted ecosystems break down the excess nitrate into N_2O, increasing total GHG emissions (Ritchie & Roser, n.d.; University of Massachusetts Amherst, 2021). A possible improved solution to these issues could be proposed by adding to our current design, creating a two-enzyme system within *O. sativa*. This new design would combine N_2OR with nitrogenase to convert N_2O into NH_3, a usable form of nitrogen for the plant, in a two-step process.

Since nitrogen has a strong triple bond and is highly chemically stable, a plant can only utilize nitrogen in its fixed, more reactive forms, such as NH_3 and nitrate (NO_3^-; Erisman et al., 2015). Such fixed forms of nitrogen are critical for optimal plant growth as this element is an essential constituent of nucleic acids, DNA, and RNA. In addition, fixed nitrogen plays a crucial role in enzyme functions that regulate water and nutrient uptake (Aczel, 2019). Nitrogenase, an enzyme commonly found in cyanobacteria and rhizobacteria, catalyzes a reaction between N_2 and hydrogen ions to form NH_3 (Esteves-Ferreira et al., 2017; Singh et al., 2020). The hypothetical transformation of *O. sativa* with N_2OR and nitrogenase would result in a plant that could eliminate the need for fertilizer, limit the environmental effects of nitrogen-based fertilizers, and alleviate agricultural costs.

We would introduce the two enzymes into *Agrobacterium tumefaciens* through a T-binary vector containing a special expression cassette containing the genes coding for both N_2OR and nitrogenase. By combining these genes within one cassette, we could introduce both enzymes into *O. sativa* using the same transformation process as before. Using a single vector reduces the risk of complications with integration patterns for multiple transformation events (Dafny-Yelin & Tzfira, 2007). However, nitrogenase is too complex for current technology and transformation methods. The *Nif* operon is too large (in terms of number of base pairs) for transformation, and past attempts have not been successful in achieving expression of a functioning enzyme. In addition, *Nif* genes can only operate in anaerobic environments, meaning that we must target transformation to the mitochondrial matrix or the chloroplast (Burén & Rubio, 2017), which can cause errors in the transformation process. Technological advancements therefore need to be made before this revised project would be feasible.

Next steps

If *Agrobacterium*-mediated transformation of *O. sativa* through the floral-dip method is successful, the next steps would involve some verification procedures that future students could perform. Beginning with PCR and RT-PCR, specific primers could be used to detect if the *nosZ* gene is present within *O. sativa*. In this procedure, RNA is extracted for reverse transcription to complementary DNA which is then used as template for PCR reactions. Further examination using gel electrophoresis will then be able to verify the presence of the *nosZ* gene. Using the Western blot technique, future students will be able to detect the presence of the N_2OR protein. Primary antibodies in the solution will bind to the protein of interest and will continue to bind, enabling visualization with a fluorescently labeled secondary antibody. Finally, students will perform the methyl viologen-linked activity assay, which takes root extract in combination with other solutions, including N_2O-saturated water in a cuvette. With the data collected by a spectrophotometer, it is possible to detect the reduction of N_2O in the solution and verify the successful function of the N_2OR protein. In the presence of methyl viologen, N_2O reduction is accompanied by concomitant oxidation of the dye, and this oxidized form can readily be detected spectrophotometrically.

Author contributions

K.L. proposed the original concept, while A.B., O.T., A.W., and J.Z. assisted in developing the idea. All authors helped research the background information required to produce the design. A.B., K.L., O.T., A.W., and J.Z. wrote and proofread the paper. The key image designers were K.L. and A.W., with insight from all other authors.

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References

Aczel, M. R. (2019, March 12). *What is the nitrogen cycle and why is it key to life?* Frontiers for Young Minds. https://kids.frontiersin.org/articles/10.3389/frym.2019.00041

Agrobacterium tumefaciens electrocompetent cells. (n.d.). Takara Bio. https://www.takarabio.com/products/cloning/competent-cells/agrobacterium-electrocompetent-cells

Aldemita, R., & Hodges, T. (1996). *Agrobacterium tumefaciens*-mediated transformation of *japonica* and *indica* rice varieties. *Planta*, *199*(4), 612–617. https://doi.org/10.1007/BF00195194

Anami, S., Njuguna, E., Coussens, G., Aesaert, S., & Van Lijsebettens, M. (2013). Higher plant transformation: Principles and molecular tools. *The International Journal of Developmental Biology*, *57*, 483–494. https://doi.org/10.1387/ijdb.130232mv

Burén, S., & Rubio, L. M. (2017). State of the art in eukaryotic nitrogenase engineering. *FEMS Microbiology Letters*, *365*(2), Article fnx274. https://doi.org/10.1093/femsle/fnx274

Chao, J. (2012, December 4). *More potent than carbon dioxide, nitrous oxide levels in California may be nearly three times higher than previously thought*. Berkeley Lab. https://newscenter.lbl.gov/2012/12/04/nitrous-oxide-levels-in-california/

Dafny-Yelin, M., & Tzfira, T. (2007). Delivery of multiple transgenes to plant cells. *Plant Physiology*, *145*(4), 1118–1128. https://doi.org/10.1104/pp.107.106104

Dangal, S. R. S., Tian, H., Xu, R., Chang, J., Canadell, J. G., Ciais, P., Pan, S., Yang, J., & Zhang, B. (2019). Global nitrous oxide emissions from pasturelands and rangelands: Magnitude, spatiotemporal patterns, and attribution. *Global Biogeochemical Cycles*, *33*(2), 200–222. https://doi.org/10.1029/2018GB006091

Devos, Y., Gaugitsch, H., Gray, A. J., Maltby, L., Martin, J., Pettis, J. S., Romeis, J., Rortais, A., Schoonjans, R., Smith, J., Streissl, F., & Suter, G. W., II. (2016). Advancing environmental risk assessment of regulated products under EFSA's remit. *EFSA Journal*, *14*, Article e00508. https://doi.org/10.2903/j.efsa.2016.s0508

Encyclopædia Britannica. (2021, July 24). Nitrogen cycle. In *Encyclopædia Britannica*. https://www.britannica.com/science/nitrogen-cycle

Erisman, J. W., Galloway, J. N., Dise, N. B., Sutton, M. A., Bleeker, A., Grizzetti, B., Leach, A. M., & de Vries, W. (2015). *Nitrogen: Too much of a vital resource*. WWF Netherlands. https://library.wur.nl/WebQuery/wurpubs/reports/488118

Esteves-Ferreira, A. A., Cavalcanti, J. H. F., Vaz, M. G. M. V., Alvarenga, L. V., Nunes-Nesi, A., & Araújo, W. L. (2017). Cyanobacterial nitrogenases: Phylogenetic diversity, regulation and functional predictions. *Genetics and Molecular Biology*, *40*(1, Suppl. 1), 261–275. https://doi.org/10.1590/1678-4685-GMB-2016-0050

Giraldo, P. A., Shinozuka, H., Spangenberg, G. C., Cogan, N. O. I., & Smith, K. F. (2019). Safety assessment of genetically modified feed: Is there any difference from food? *Frontiers in Plant Science*, *10*, Article 1592. https://doi.org/10.3389/fpls.2019.01592

Greenhouse gas sources and sinks. (n.d.). American Chemical Society. https://www.acs.org/content/

acs/en/climatescience/greenhousegases/sourcesandsinks.html

Harper, D. (2022, July 7). *Plant transformation using Agrobacterium tumefaciens*. African Biosafety Network of Expertise. https://nepad-abne.net/process-of-developing-genetically-modified-gm-crops/plant-transformation-using-agrobacterium-tumefaciens/

Hergert, G., Nielsen, R., & Margheim, J. (2015, April 10). *WWII nitrogen production issues in age of modern fertilizers*. University of Nebraska–Lincoln. https://cropwatch.unl.edu/fertilizer-history-p3

Hulse, M., Johnson, S., & Ferrieri, P. (1993). *Agrobacterium* infections in humans: Experience at one hospital and review. *Clinical Infectious Diseases*, *16*(1), 112–117. https://doi.org/10.1093/clinids/16.1.112

Hwang, H.-H., Yu, M., & Lai, E.-M. (2017). *Agrobacterium*-mediated plant transformation: Biology and applications. *The Arabidopsis Book*, *15*, Article e0186. https://doi.org/10.1199/tab.0186

Kámán-Tóth, E., Pogány, M., Dankó, T., Szatmári, Á., & Bozsó, Z. (2018). A simplified and efficient *Agrobacterium tumefaciens* electroporation method. *3 Biotech*, *8*, Article 148. https://doi.org/10.1007/s13205-018-1171-9

Kanter, D. R., Zhang, X., Mauzerall, D. L., Malyshev, S., & Shevlikova, E. (2016). The importance of climate change and nitrogen use efficiency for future nitrous oxide emissions from agriculture. *Environmental Research Letters*, *11*, Article 094003. https://doi.org/10.1088/1748-9326/11/9/094003

Kroemer, T. (n.d.). *A guide to T-DNA binary vectors in plant transformation*. GoldBio. https://www.goldbio.com/articles/article/a-guide-to-agrobacterium-t-dna-binary-vectors

LBA4404 electrocompetent Agrobacterium. (n.d.). Intact Genomics. https://www.intactgenomics.com/product/lba4404-electrocompetent-agrobacterium/

Messerschmidt, A. (2010). Copper metalloenzymes. In L. N. Mander, H.-W.-Liu, & C. P. Whitman (Eds.), *Comprehensive natural products II: Chemistry and biology. Vol. 8: Enzymes and enzyme Mechanisms* (pp. 489–545). Elsevier.

Mikkelsen, R. (n.d.). *Understanding fertilizer and its essential role in high-yielding crops*. Mosaic. https://www.cropnutrition.com/resource-library/understanding-fertilizer-and-its-essential-role-in-high-yielding-crops

Nester, E. W. (2015). *Agrobacterium*: Nature's genetic engineer. *Frontiers in Plant Science*, *5*, Article 730. https://doi.org/10.3389/fpls.2014.00730

Nielsen, K. M., & Daffonchio, D. (2007). *Unintended horizontal transfer of recombinant DNA* (TWN Biotechnology & Biosafety Series 13). Third World Network. https://genok.no/wp-content/uploads/2013/04/Chapter-13.pdf

Nucleotide [Internet]. (2022, May 22). *Bradyrhizobium diazoefficiens USDA 110, complete sequence* (Accession No. NC_004463.1). Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information. https://www.ncbi.nlm.nih.gov/nuccore/NC_004463

Peralta, E. G., & Ream, L. W. (1985). T-DNA border sequences required for crown gall tumorigenesis. *Proceedings of the National Academy of Sciences*, *82*(15), 5112–5116. https://doi.org/10.1073/pnas.82.15.5112

Petrunia, I. V., Frolova, O. Y., Komarova, T. V., Kiselev, S. L., Citovsky, V., & Dorokhov, Y. L. (2008). *Agrobacterium tumefaciens*-induced bacteraemia does not lead to reporter gene expression in mouse organs. *PLoS One*, *3*(6), Article e2352. https://doi.org/10.1371/journal.pone.0002352

Pomowski, A., Zumft, W. G., Kroneck, P. M. H., & Einsle, O. (2011). N_2O binding at a [4Cu:2S] copper–sulphur cluster in nitrous oxide reductase. *Nature*, *477*, 234–237. https://doi.org/10.1038/nature10332

Quinn, R. (2021, December 15). *DTN retail fertilizer trends*. Progressive Farmer. https://www.dtnpf.com/agriculture/web/ag/crops/article/2021/12/15/nitrogen-fertilizer-prices-continue

Ritchie, H., & Roser, M. (n.d.). *World population supported by synthetic nitrogen fertilizers*. Our World in Data. https://ourworldindata.org/grapher/world-population-supported-by-synthetic-nitrogen-fertilizers?country=~OWID_WRL

Sedlacek, C. J., Giguere, A. T., & Pjevac, P. (2020, May 20). *Is too much fertilizer a problem?* Frontiers for Young Minds. https://kids.frontiersin.org/articles/10.3389/frym.2020.00063

Shankman, S. (2019, September 11). *What is nitrous oxide and why is it a climate threat?* Inside Climate News. https://insideclimatenews.org/news/11092019/nitrous-oxide-climate-pollutant-explainer-greenhouse-gas-agriculture-livestock/

Singh, R. K., Singh, P., Li, H.-B., Song, Q.-Q., Guo, D.-J., Solanki, M. K., Verma, K. K., Malviya, M. K., Song, X.-P., Lakshmanan, P., Yang, L.-T., & Li, Y.-R. (2020). Diversity of nitrogen-fixing rhizobacteria associated with sugarcane: A comprehensive study of plant-microbe interactions for growth enhancement in *Saccharum* spp. *BMC Plant Biology*, *20*, Article 220. https://doi.org/10.1186/s12870-020-02400-9

Suter, G., II, Cormier, S., Schofield, K., Bowersox, M., & Latimer, H. (2022, March 21). *Ammonia*. United States Environmental Protection Agency. https://www.epa.gov/caddis-vol2/ammonia

United States Environmental Protection Agency. (2022, May 16). *Overview of greenhouse gases*. https://www.epa.gov/ghgemissions/overview-greenhouse-gases

University of Massachusetts Amherst. (2021, October 19). *Agricultural runoff contributes to global warming – new study helps us figure out how and what we can do about it*. ScienceDaily. https://www.sciencedaily.com/releases/2021/10/211019110540.htm

U.S. Energy Information Administration. (2011, March 31). *Emissions of greenhouse gases in the U.S.* https://www.eia.gov/environment/emissions/ghg_report/tables_ghg.php

Wallach, O. (2022, March 9). *This is how much rice is produced around the world – and the countries that grow the most*. World Economic Forum. https://www.weforum.org/agenda/2022/03/visualizing-the-world-s-biggest-rice-producers/

Wan, S., Johnson, A. M., & Altosaar, I. (2011). Expression of nitrous oxide reductase from *Pseudomonas stutzeri* in transgenic tobacco roots using the root-specific *rolD* promoter from *Agrobacterium rhizogenes*. *Ecology and Evolution*, *2*(2), 286–297. https://doi.org/10.1002/ece3.74

Warwick, S. I., Beckie, H. J., & Hall, L. M. (2009). Gene flow, invasiveness, and ecological impact of genetically modified crops. *Annals of the New York Academy of Sciences*, *1168*(1), 72–99. https://doi.org/10.1111/j.1749-6632.2009.04576.x

Xu, C., Li, L., Jin, W., & Wan, Y. (2014). Recombinase polymerase amplification (RPA) of CaMV-35S promoter and *nos* terminator for rapid detection of genetically modified crops. *International Journal of Molecular Sciences, 15*(10), 18197–18205. https://doi.org/10.3390/ijms151018197

Zhang, L., Wüst, A., Prasser, B., Müller, C., & Einsle, O. (2019). Functional assembly of nitrous oxide reductase provides insights into copper site maturation. *Proceedings of the National Academy of Sciences*, *116*(26), 12822–12827. https://doi.org/10.1073/pnas.1903819116