Increasing wheat protein concentration by promoting *narB* expression

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Malnutrition affects over two billion people worldwide. Among the various forms of malnutrition, protein-energy malnutrition disproportionately affects children. As of 2019, 52 million children under the age of five suffer from low protein intake. Two major causes of protein-energy malnutrition are the relatively high cost and low availability of high protein foods. In areas that suffer most from malnutrition, most of the population cannot afford nutritious foods or do not have access to healthier options. As a result, many can only subsist on diets heavy in carbohydrates as their health remains chronically suboptimal due to an imbalance of macronutrients. To remedy this problem, we designed a system aimed at increasing the protein content of common wheat. Wheat is a cheap grain widely available in many areas of the world, including those most impacted by malnutrition. The system will use *narB*, a gene naturally found in most crops which codes for the enzyme *nitrate reductase.* Responsible for reducing nitrate from fertilized soil to nitrite, nitrate reductase is critical to the production of protein in most plants, including wheat. A disarmed Ti plasmid containing the narB gene under the control of the β-conglycinin promoter will be inserted via electroporation into embryonic *Triticum aestivum* (common wheat) cells. β-conglycinin is a well-characterized embryo-specific promoter naturally found in soybeans that will be used to enhance the expression of *narB* in wheat. We use a disarmed Ti plasmid because of its ability to integrate into the host genome. These plasmids are commonly used in *Agrobacterium*-mediated plant gene transfer. Ideally, the insertion of the plasmid into wheat embryos will allow for both an increased nitrogen uptake efficiency and protein content in the modified wheat crops. Upon maturing, each transformant's protein content will be measured to evaluate the effectiveness of our design. A successful implementation of this technique may provide a solution to the widespread protein-energy malnutrition among children in developing countries.

Keywords: Increased protein, *agrobacterium tumefaciens*, wheat, *triticum aestivum*

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Watch a video introduction by the authors at https://youtu.be/aQAYD8Msqnw

Background

Protein-energy malnutrition (PEM) is responsible for millions of deaths annually in children and adults worldwide (Fantuz, Papademas & Salimei, 2016). Though PEM also occurs in industrialized nations, it is far more prevalent in developing countries where the scarcity of food and a lack of nutritional options force people to live on inadequate diets (Ahmed et al., 2020). PEM symptoms include stunted growth, weight gain, behavioral shifts and are a common cause of secondary immunodeficiency among children (Ahmed et al., 2020). Additionally, low bone density and muscle atrophy, which increase the likelihood of bone fractures, are common in developing countries and are one of the primary symptoms of a low protein diet (Young & Pellett, 1985).

To combat this problem, our team selected *Triticum aestivum* (common wheat) for its prevalence across the globe. Wheat accounts for close to 17% of the world's cultivated land. Of the various proteins found in *Triticum aestivum*, *Glutenin* and *Gliadin* make up nearly 50%. Currently, a diet subsisting solely on wheat as a source of protein is not viable (Fantuz, Papademas, &Salimei, 2016). Higher protein levels in wheat require an excess of nitrogen in the soil, an essential element for creating amino acids (Hawkesford, Ludewig, & Zörb, 2018). Excess Nitrogen is often supplied by the fertilizer farmers use to increase the yield and protein levels of their crops (Hawkesford, Ludewig, & Zörb, 2018). For wheat, the process of nitrogen assimilation begins with *nitrate reductase*. *Nitrate reductase* converts the Nitrate (NO_3) that a plant absorbs into Nitrite (NO_2) (Bucher & Kossmann, 2007). Next, nitrite reductase reduces the Nitrite (NO_2) to ammonium (NO), where it can be incorporated into amino acids for protein synthesis (Iwata, Yazaki, Yoneyama, 1980).

Our proposed design will increase the expression of the *narB* gene to supply wheat with the excess nitrogen needed to grow and produce essential amino acids. Our team aims to increase the protein content of *Triticum aestivum* to tackle the problem of PEM at its source. Our objective is to make wheat a reliable protein source in places where protein-rich foods are inaccessible.

Systems Level

Our team's design will utilize the binary *Agrobacterium* vector pBI121 to create transgenic plants (refer to figure 1). We chose to use a strain of *Agrobacterium tumefaciens* for its effectiveness at transferring and integrating modified DNA with a plant's DNA (Gelvin, 2003). A disarmed Ti-plasmid is a plasmid with a deleted tumor-inducing gene (Gelvin, 2003). Firstly, the gene *narB,* which codes for the *molybdoenzyme nitrate reductase,* would be cloned. A β-conglycinin βeta subunit 2 promoter will also be inserted into the cut disarmed *Ti-plasmid* to increase the expression of the *narB* gene.

The second step of this process is transferring the plasmid containing the gene of interest into a plant embryo. For this stage, we used electroporation for transference. Electroporation is the process of using an electrical charge to make a cell's membrane more permeable (Tsong, 1991). The increased permeability allows for a *Ti-plasmid* to be introduced and integrated with the DNA of wheat cells. Lastly, it must be determined if the DNA is successfully integrated with the chromosome of the wheat. PBI121 is resistant against both Kanamycin and Neomycin (GSL Biotech LLC, 2020). If successful, protein concentration can be measured using near-infrared spectroscopy to determine whether the concentration of protein is higher or lower than expected (Zörb, 2018).

Device Level

Our design utilizes the binary *Agrobacterium* vector pBI121 for wheat embryo transformation. Agrobacterium-mediated plant transfer is both effective and reproducible in wheat, a cereal typically difficult to transform (Hayta et al., 2019). Cloning in *narB* and the β-conglycinin β subunit gene into the T-DNA regions of

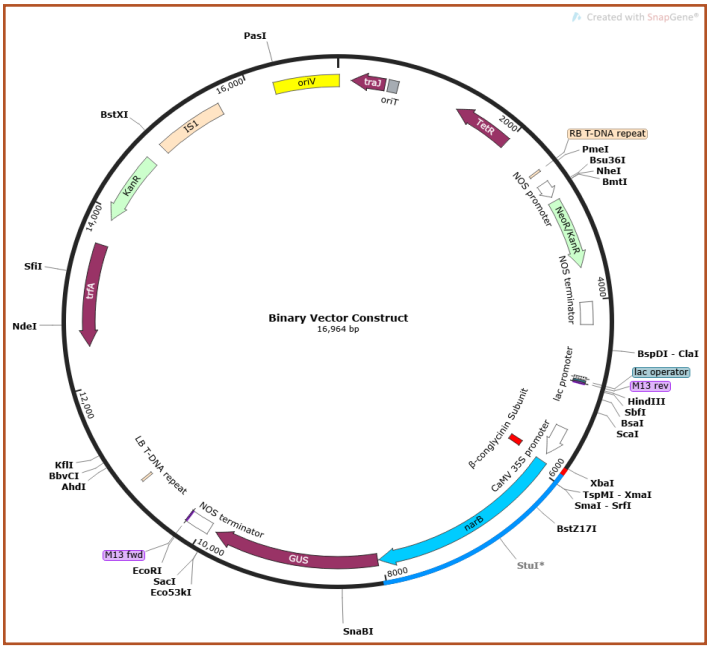

Figure 1. *Modified binary vector construct. Construct is synthesized from pBI121 and the cloning of narB and the β-conglycinin β subunit gene.*

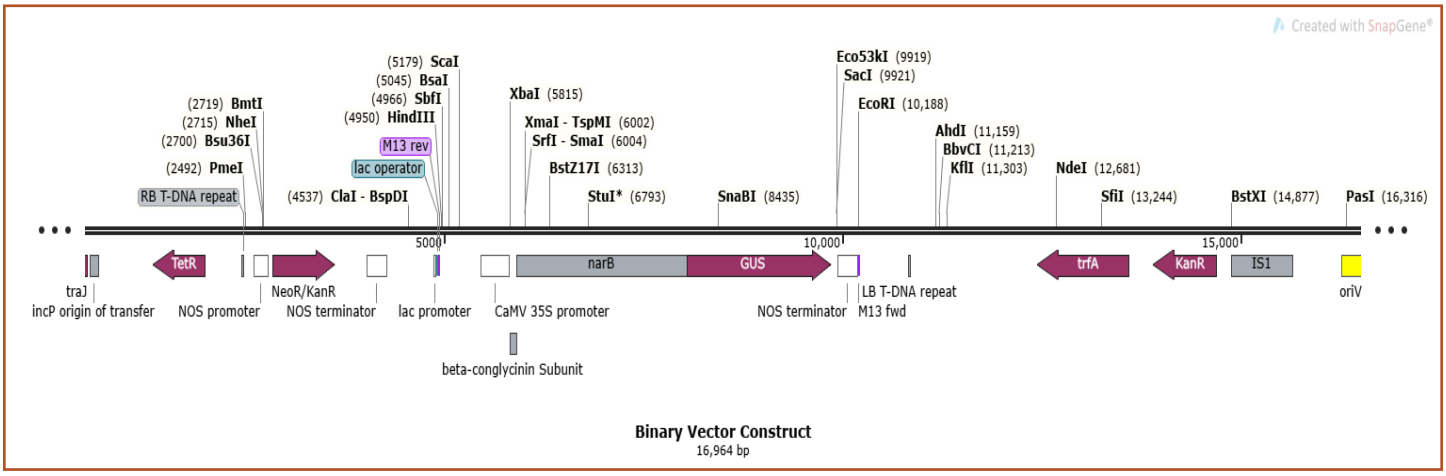

Figure 2. *Diagram of biological parts. Both the narB gene as well as the β-conglycinin β subunit gene will be under the control of the CaMV 35S promoter and the NOS terminator.*

pBI121, enables transference through electroporation. The virulent helper gene pBI121 allows for the replication of the T-DNA within the genome of the wheat embryo. Additionally, pBI121 contains the *GUS* reporter gene (GSL Biotech LLC, 2020). The GUS reporter gene produces a blue stain in plants when successfully integrated (Basu, 2004).

In successfully integrated plants, we believe that the overexpression of Nitrate reductase will induce the production of Nitrite reductase. If this occurs, the nitrite should naturally be reduced to ammonia, where it can integrate with amino acids to increase the overall protein concentration.

Parts Level

The binary vector construct consists of *narB* and the β-conglycinin β subunit gene cut at the *SmaI* restriction site (See figure 2). The two fragments can be ligated at the *NsiI* restriction site where the sticky end can then be blunted.

Safety

Through studies on the use of Nitrogen fertilizers, it was discovered that an accumulation of nitrate can be toxic to humans and other animals (Likens et al., 2002). Similarly, nitrite can also be toxic in excess to both herbivores and carnivores (Likens et al., 2002). Testing our design outside of a lab setting could pose the risk of surface water and drinking water contamination since sodium nitrate and sodium nitrite dissolve in water. In the US, drinking water must have less than 10 mg/L of both nitrite and nitrate for it to be considered safe for non-infants (United States Environmental Protection Agency, 2021).

We believe that the risk of nitrate toxicity is low due to the increased expression of the narB gene which would prevent a harmful buildup of nitrate. In the case that the wheat exceeds the safety standard for nitrite, our team believes that cloning in NirS into our disarmed Ti plasmid will mitigate risks of nitrate toxicity. NirS is the gene that encodes for the enzyme *nitrate reductase* (Dong, Nedwell, Osborn, & Smith, 2007). An increased expression of nitrite reductase would manage the excess nitrates produced by the overexpressed narB gene. Overall the team deemed the risk of nitrate contamination low as it is most often due to fertilizers (Water Education Foundation, 2020). Another potential risk would come to those with gluten sensitivity and celiac disease. In theory, our proposed design would contain more of the proteins glutenin and gliadin normally found in wheat which might result in a more acute immune response in comparison to normal wheat.

Discussions

One challenge for us has been distance learning which has rendered us unable to test out any aspect of our design in a lab setting. Another challenge was finding the promoter region of β-conglycinin's β subunit gene. To solve this, we made an approximation of where it would be located. However, we plan on doing more research into β-conglycinin. In the future we plan on improving our plasmid design to minimize risks and improve its effectiveness. One of these improvements would be the addition of a ribosome binding site that we had trouble locating in binary vector pBI121. Another issue we faced

early on was deciding on the method for transferring our plasmid. We eventually settled on electroporation since the equipment and reagents were more accessible to us.

Next Steps

Until we can perform the experiments ourselves at our school's lab, we plan on putting in more research into the possible impacts our design could have on the environment. We recognize that without the proper precautions, our design could potentially be a harm to our community and local wildlife, so we plan on thoroughly reviewing any safety risks. We also plan on researching more about our gene of interest as well as the β subunit promoter of β-conglycinin in order to improve upon our design.

Author Contributions

T.M. created the idea and did the initial research to determine whether our design was probable. T.M. also suggested the gene of interest (*narB*) and the β-conglycinin promoter used to increase the expression of the gene being studied. A.O. created the plasmid design and assisted with the research process. A.O. also identified the possible research design flaws and safety hazards which helped guide the research process.

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