Design Brief

Maximizing protein and glucosinolate content in *Brassica oleracea*: the ultimate broccoli*

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The aim of this project is to enhance the nutritional content and yield of Brassica oleracea (broccoli) through genetic engineering. Broccoli is known for its large quantity of essential vitamins such as C, K, and A and minerals such as potassium, calcium, and iron, as well as antioxidants such as vitamins C and E, and β-carotene. Broccoli is rich in anti-inflammatory, anti-carcinogenic nutrients. However, despite having relatively significant protein content at 2.6 g per 100 g, the nutritional effects of protein and glucosinolate in broccoli are not as evident. Hence, our focus is on mofidying the protein and glucosinolate composition of broccoli to increase their content and nutritive effects. We intend on using CRISPR-Cas9 technology to maximize expression of two genes in Green King (GK) broccoli: MYP34 that controls the production of aliphatic glucosinolates, and CYP79A2 that transforms amino acids into aldoximes, which are nitrogen-rich compounds. Secondly, we aim to conduct cross-plant genetic engineering with Chinese kale to introduce the BoMYB29 gene, which is a key regulator of the biosynthesis of methylsulphinyl glucosinolate that is rich in amino acids. We also aim to insert the serine/threonine protein kinase SRK2n(SnRK2) gene.

Keywords: Broccoli genes, protein synthesis, gene expression, nutrition enhancement

The global challenge of malnutrition persists, notably within socioeconomically disadvantaged areas characterized by constrained access to nutrient-dense sustenance, whereby protein inadequacy emerges as a prominent concern that impedes child development and well-being. Broccoli (*Brassica oleracea)*, one of the most affordable candidates, serves an exceptional nutritional profile with abundance in essential vitamins, minerals, and antioxidants (Syed R et al, 2023; "*Top 9 Cheapest and Healthiest Green Veggies", 2023)*. However, its relatively modest protein content creates room for improvement. Recognizing broccoli's potential as an accessible and cost-effective source of nutrition, this project aims to elevate its protein content through genetic modification.

The utilization of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) genetic editing targets specific genes within the broccoli genome. Our focus lies on enhancing the expression of MYP34 and CYP79A2 genes, known to influence protein and glucosinolate levels respectively, we aim to augment the nutritional profile of broccoli while preserving its inherent health benefits (Miao H. *et al*, 2021; Yi GE *et al*, 2016). In addition to internal modifications, crossbreeding with Chinese kale introduces the BoMYB29 gene and serine/threonine

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protein kinase SRK2n (SnRK2) genes into the broccoli genome. These genes, associated with enhanced protein synthesis and stress tolerance, respectively, contribute to the development of a more resilient crop capable of thriving in diverse environmental conditions.

Systems level

This study explores the complex systems-level tactics used to raise the protein and glucosinolate content of *B. oleracea*, with a particular emphasis on broccoli. As a member of the Brassicaceae family, *B. oleracea* is highly valued for its abundant nutritional content, particularly for its high protein and glucosinolate levels, which offer numerous health advantages. To satisfy changing consumer demands, there is an increasing need for cultivars with progressively greater nutritional profiles. This study optimizes the protein and glucosinolate content of broccoli cultivars by a multimodal strategy that integrates genetic selection, breeding techniques, and agronomic practices.The goal of the research is to enhance the protein content of broccoli by modifying its genes and elevate production of glucosinolate by cross-breeding with Chinese kale, advancing its value as a nutritive “superfood”.

This information forms the basis for creating focused strategies. In addition, sophisticated molecular methods like genome editing and marker-assisted selection are applied to speed up the breeding process and precisely introduce desirable features. Through the application of state-of-the-art biotechnological instruments, the study aims to expedite the creation of broccoli cultivars that are superior in terms of nutrition. The main objective of this research is to help develop broccoli cultivars that have higher levels of protein and glucosinolates while retaining their conventional nutritional advantages. These cultivars may have increased health-promoting qualities, including enhanced antioxidant activity, anti-inflammatory benefits, and possibly even cancer-preventive qualities. This research aims to improve the nutritional status of customers by meeting their unique needs through focused breeding efforts.

Device level

The CRISPR-Cas9 system offers an effective approach for targeted gene editing in *Brassicaceae* crops, allowing for the manipulation of genes associated with desired traits. Here, we explore the application of CRISPR-Cas9 for editing four genes of interest: MYB34, CYP79A2, BoMYB29, and SnRK2n.

Firstly, in order to activate the MYB34 gene, CRISPR-Cas9 would need a specially created guide RNA (gRNA) that would target a particular regulatory region within the MYB34 coding sequence. This region is essential for regulating the activity of MYB34. The cell's normal DNA repair processes would then try to mend the double-strand break (DSB) that was created at this target location. In some cases, this can introduce mistakes (insertions or deletions) that alter the function of the MYB34 protein and cause its permanent activation.

Secondly, to activate the CYP79A2 gene. The gRNA would specifically target the area of the CYP79A2 coding sequence that is in charge of the enzyme's activity. Cellular repair mechanisms would once again be triggered upon Cas9-induced DSB at this site. Rather than completely disrupting the enzyme, gRNAs that induce targeted mutations that increase the enzyme's activity could be created. This exact alteration has the ability to greatly increase glucosinolate content without impairing other cellular functions.

In addition, with respect to the BoMYB29 gene, the CRISPR-Cas9 system would function differently. It would be used for selective insertion of the complete BoMYB29 gene rather than causing induced mutations. The gRNA would be designed to target a specific location in the broccoli genome suitable for gene insertion. DNA polymerase is another enzyme that would be added in addition to Cas9. To properly insert the functional BoMYB29 gene sequence at the desired chromosomal region, this enzyme would make use of a donor DNA template. The design of the gRNA is essential for choosing the proper insertion site within the

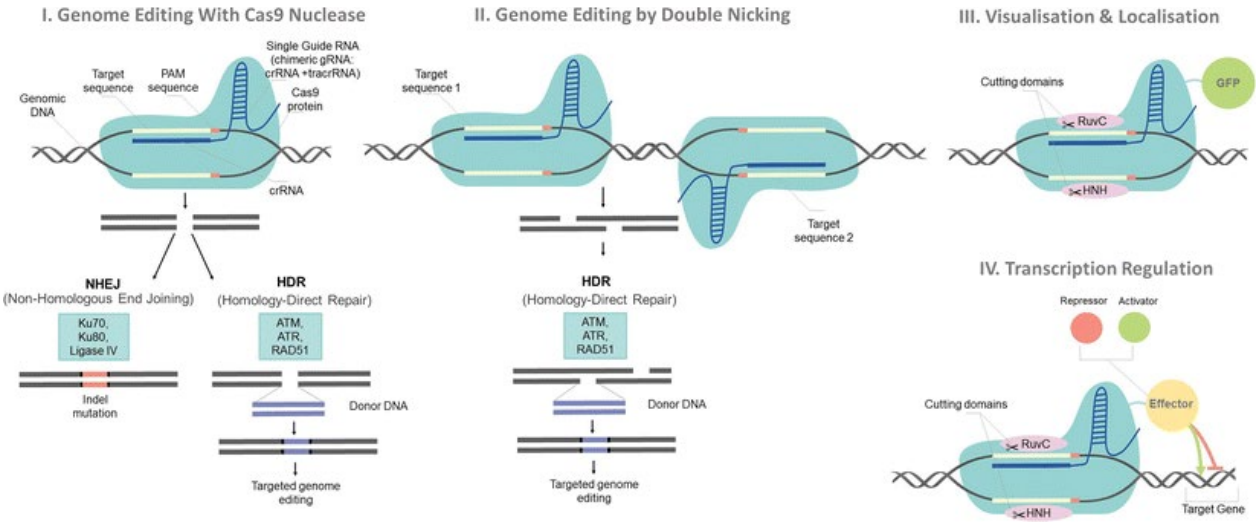

Figure 1. CRISPR-Cas9 genetic engineering procedures (Grzybek, n.d.)

broccoli genome, even though the gRNA itself wouldn't target the sequence of the BoMYB29 gene.

Last but not least, for the SnRK2n gene, CRISPR-Cas9 would be used for gene insertion, just like it was with BoMYB29. A specific region of the *B. oleracea* genome would be the target of the gRNA, and Cas9 would produce a DSB. DNA polymerase would then make it easier for the new gene to be precisely inserted, increasing the plant's resistance to stress, along with a donor DNA template that contained the SnRK2n gene sequence. The gRNA sequence itself, similar to BoMYB29, would target a specific insertion site within the *B. oleracea* genome rather than the SnRK2n gene.

Parts level

In the Parts Level section, an explanation of advanced genetic engineering tools utilized to refine the genetic composition of *B. oleracea* (broccoli) is provided. A fundamental technique employed is CRISPR technology, renowned for its precision in DNA sequence modifications (Ormond et al, 2017). With CRISPR, targeted alterations such as additions, deletions, or modifications of genetic sequences within plant genomes can be achieved, facilitating enhancements in desired traits, including increased nutrient content and enhanced resilience to environmental stressors ("CRISPR-Cas9 and plant genetic engineering," n.d.)

Furthermore, alongside CRISPR, other techniques are leveraged for the construction of genetic vectors known as plasmids. These plasmids serve as carriers for introducing desired genetic material into plant cells, and will be delivered using gel electroporation ("CRISPR-Cas9 and plant genetic engineering," n.d.). Once integrated, these genetic instructions facilitate desired growth patterns and trait expressions within the plants. Additionally, meticulous analysis of plant DNA and RNA discussed below, such as colorimetric dye-based protein assays, will be conducted to validate the efficacy of genetic modifications. This analysis ensures the accurate insertion and expression of target genes within plant genomes.

Incorporating insights from recent research articles on synthetic biology, such as advancements in CRISPR/Cas9 gene editing technology (Crown Bioscience, 2024), the application of advanced genetic engineering tools in synthetic biology has garnered considerable attention. The CRISPR/Cas9 system represents a cutting-edge gene editing platform that enables precise and efficient modification of a cell's DNA (Crown Bioscience, 2024). This revolutionary technology has significantly impacted drug discovery endeavors by simplifying editing processes and minimizing off-target effects (Crown Bioscience, 2024). With

comprehensive services covering in vitro and in vivo efficacy studies, including disease modeling using stable knockouts/knock-ins and testing drug efficacy, researchers can confidently model diseases and evaluate the effectiveness of therapeutic interventions (Crown Bioscience, 2024).

As seen in Figure 1, CRISPR-Cas9 involves inducing the repair mechanism of DNA and RNA. Homology-Direct Repair (HDR) involves the Cas9 protein forming a Cas9-RNA complex with Single Guide RNA, which attaches to the target sequence of genomic DNA with a short PAM sequence which is necessary for the incision of DNA (Ding et al, 2023; Wan et al, 2021). The attached Cas9-RNA complex slices through the PAM sequence, cutting through the double helix (Ball, 2016). HDR includes insertion of donor DNA with the selected genes previously elucidated in the device level section in the gap (Grzybek, 2018). As hydrogen and phosphodiester bonds form, the newly inserted gene will become part of the new DNA sequence.

Once broccoli cells have been modified with the respective genes, a range of checking methodologies can be utilized to test for any mutations or unsuccessful modifications, and whether the protein content has been accordingly modified. The major assessment will be sequencing analysis in DNA:PCR amplification and gel electrophoresis will be run if genetic modification with CRISPR has taken place in the engineered broccoli cells. As secondary analyses, biomass quantification may be conducted in the following sequence: pelleting the cells out, acid/base pre-treatment, enzyme treatment to break down sugars and complex organic structures, centrifugation and filtration to separate sugars from the core nutrients including glucosinolate, and lastly running colorimetry for protein quantification. Lastly, to assess whether there are any modifications such as insertions or deletions at unintended sites, off-target analysis can be performed and elevate the specificity of the CRISPR-Cas9 system. With the modified cell pellet, the most potential techniques are whole genome sequencing (WGS) as cell-based off-target analysis or *in vitro* digenome sequencing by digesting gDNAs with ribonucleoproteins and performing WGS (Lopes & Prasad, 2024). Through this, the population of successfully edited cells may be expanded for further experimentation or downstream applications.

Another methodology that can be used to quantitatively measure and contrast the effectiveness of the procedures is measuring reactive oxygen species (ROS) content. ROS are oxygen radicals with a single unpaired electron. This characteristic amplifies the reactivity of radicals as they readily donate their unpaired electrons and act as oxidizing agents (Traxler et al, 2023). Glucosinolate reduces oxidative stress by inducing enhanced scavenging ability of ROS, which was explored in an investigation where the *Brassica rapa* cells with high GSL contents (HGSL) effectively reduced oxidative stress and improved cell viability of lipopolysaccharide (LPS)-stimulated RAW264.7 cells from a tumor in male mouse (Choi et al, 2023). Hence, ROS content in the engineered broccoli can be determined through histochemical/immunohistological staining techniques, such as 3,3′-Diaminobenzidine (DAB) staining to determine hydrogen peroxide content. Superoxide accumulations can be observed qualitatively with a microscope, or quantitatively through spectrophotometry on colloidal suspensions containing the modified broccoli cells (“3,3’-Diaminobenzidine tetrahydrochloride hydrate”, 2017; Meena et al., 2016).

Safety

The usage of CRISPR-Cas9 technology requires considerable planning to ensure the most effective usage of this technology, reducing the amount of off-target effects gained from it. For example, choosing the right guide DNA to prevent it from guiding to the wrong part of the DNA, and carefully adjusting the experimental conditions to have the CRISPR-Cas9 technology deliver its results in an optimum state, such as determining the optimal concentration of the gRNA-Cas9 complex and HDR template (Vorobev, 2023).

As in all experiments, regular experimental procedures must also be followed, such as wearing lab coats, eye

protection, and hand gloves, to prevent any excessive injury from accidents.

Discussions

This design brief implements the usage of CRISPR-Cas9 technology, which is highly sophisticated as the Cas9 enzyme locates the specific target gene in donor DNA via guide RNA to cleave that target in the desired location (Strack, 2018). The post-modification analyses and assays are also thorough and comprehensive, employing sequencing analysis in DNA, colorimetry, gel electrophoresis, biomass quantification via acid/base pre-treatment, and off-target analysis, which reduces the potential risk of errors in the modified genes inside the broccoli. On the one hand, some challenges and limitations faced include the lack of feasibility to conduct all expected assays and analyses. For instance, quantitative PCR (qPCR) is done by converting the subject mRNA into complementary DNA (cDNA), then analyzing it using a PCR machine quantitatively via fluorescence (Temple, 2021). qPCR has been proven useful in providing fast and high-throughput detection and quantification of target DNA sequences in different matrices (Kralik & Ricchi, 2017). However, due to the high cost and scarcity of resources, qPCR is low in feasibility to be applied as one of the testing methods. Furthermore, the topic of genetically modified foods may raise ethical concerns towards the safeness for consumption and chances of unknown health issues that may arise due to hidden errors in gene insertion during the CRISPR editing process or due to poor cell culture management and sterility (Wan L et al, 2021).

Next steps

To further advance the objectives outlined in this project, gene expression analysis via RT-PCR assays will quantify the expression levels of targeted genes in transformed broccoli tissues compared to wild-type controls, elucidating the impact of gene editing on protein and glucosinolate levels. To complement the molecular analyses, physiological studies will be done to assess the impact of genetic modifications on broccoli growth and development, investigating parameters including plant morphology, flowering patterns, and seed yield. Growth performance and stress tolerance of genetically modified broccoli plants will be evaluated under varying environmental conditions, monitoring key parameters including yield and biomass. This provides valuable insights into their agronomic potential. Additionally, collaboration will be fostered with local agricultural experts or community gardens to conduct field trials of the genetically modified broccoli under real-world conditions. Organizing outreach events or workshops in collaboration with community gardens can facilitate knowledge-sharing about the science behind genetic engineering and its implications for agriculture and food security.

Author contributions

J.K. was leader of the project, initiated meetings, assigned roles, divided parts, identified genes, researched post-modification analysis, and researched the CRISPR-Cas9 technique. Y.C. explored the impact of the project and targeting the problem, and was treasurer. J.X. identified genes, researched post-modification analysis, and was secretary. A.Tan researched pros and cons of the project and the “safety” section of the report, and produced figures and blueprints in the planning process. A.Tyagi researched the CRISPR-Cas9 technology, and explored genetic engineering for the Parts Level. C.D. explored post-modification analysis techniques and was vice president.

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