Research Article

Effect of Myrosinase Enzyme Encoding Gene Knockout on the Bitter Taste of Broccoli: A CRISPR-Cas9 Experimental Proposal

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ABSTRACT

Many people perceive bitterness when consuming broccoli. Glucosinolates and their degraded products are the main contributors to the bitter taste. Production and hydrolysis of glucosinolates require c gene using CRISPR-Cas9 will reduce broccoli's bitter taste. This study also hypothesized that knocking out the myrosinase encoding gene will reduce bitterness in broccoli. The research involves designing a plasmid to deliver the CRISPR-Cas9 system to target and disrupt broccoli's myrosinase enzyme encoding gene. Successful completion of this proposal could lead to the development of a broccoli variety that is more palatable to a broader population, potentially increasing its consumption and associated health benefits.

Keywords: CRSIPR-Cas9; biochemistry; gene knockout; broccoli; gene editing; glucosinolates

INTRODUCTION

Many people experience a bitter taste when consuming broccoli (*Brassica oleracea var. italica*). This bitterness is related to the human gene for bitter receptors named TASR38 (1). Broccoli contains glucosinolates, a major secondary metabolite, which, upon contact with the enzyme myrosinase, undergoes hydrolysis to produce various breakdown products (2). The breakdown product typically includes isothiocyanates which give broccoli a bitter tasting flavour (3). Glucosinolates and isothiocyanates contribute to the bitter taste in most Brassicaceae family plants, with myrosinase enzymes catalyzing this degradation process(4, 5). This mechanism has evolved in these plants as a defence mechanism against pests (6). Glucosinolates have also exhibited protective activities against inflammation and diseaseses such as cancer (7).

This study proposes a method to knock out a myrosinase enzyme encoding gene in broccoli using CRISPR/Cas9 technology. The approach employs bioinformatics tools to identify the specific target sequence of the myrosinase gene in broccoli, followed by the design of guide RNAs (gRNA) and genotyping primers specific to this sequence. The experiment aimed to deliver the CRISPR-Cas9 system into the broccoli plant via *Agrobacterium*-mediated transformation to target and knock out a myrosinase encoding gene. This technique relies on an engineered plasmid which can be transferred from the *Agrobacterium* to the plant cell. The essential part of the plasmid for engineering is the Transfer DNA (T-DNA), which will be integrated into the plant genome. The T-DNA must contain these elements:

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- A promoter driving the Cas9 enzyme sequence
- Promoters driving gRNA sequences
- A promoter driving a Selection marker for identifying transformed plants
- Left border (LB) & Right border(RB) sequences for helping integrate the T-DNA into the plant genome.

The CRISPR-Cas9 system is a tool that can be used for many purposes including gene knockout, inserting sequences into genomes, and gene modification (8). The CRISPR-Cas9 system consists of two main components, the single guide RNA (sgRNA), and the Cas9 enzyme. The sgRNA includes two parts, a crRNA which is the customizable part to target specific gene sequences, and the scaffold tracrRNA sequence. For the Cas nuclease to bind with the target sequence, a protospacer adjacent motif (PAM) must be located 3-4 base pairs downstream of the cutting location (8). The Cas9 nuclease will make a double-strand break (DSB). After the DSB occurs, DNA-repair machinery will try to fix the DNA through one of the two DNA repair methods. One common method for DNA repair is non-homologous end joining (NHEJ), which directly ligates the two ends together. Occasionally, mutations happen during the ligating process, that disrupt the normal reading frame for amino acids, which results in a frameshift mutation (9). In this experiment, *Agrobacterium* was used to carry the plasmid, which contains an LB and an RB to integrate the T-DNA into the plant genome. The CRISPR tool in the T-DNA then facilitates DSBs causing frameshift mutation therefore knocking out the target gene (10).

Given that glucosinolates' degradation products are the main source of bitterness for Brassicaceae plants and myrosinase is essential for the degradation process. This study hypothesized that knocking out the myrosinase encoding genes would reduce or eliminate broccoli's bitter taste. However, this article will focus on outlining the initial design, methodology, and theoretical framework of the proposed experiment, without delving into further experimental results or subsequent procedural steps.

METHODS

Sequence identification

To design the CRISPR guide RNA, the sequence of the myrosinase enzyme encoding gene was identified in broccoli. Although there is a complete sequenced genome for broccoli, the Myrosinase enzyme encoding gene has not yet been functionally annotated. However, because broccoli is closely related to *Arabidopsis thaliana*. It is hypothesized that broccoli has an ortholog to the *Arabidopsis* myrosinase enzyme encoding gene. To accurately identify the myrosinase enzyme encoding gene in *Brassica oleracea var. italica* (broccoli), bioinformatics tools were employed to confirm gene orthology with *Arabidopsis thaliana*, a closely related species in the Brassicaceae family.

BLAST Analysis. The myrosinase gene sequence from *Arabidopsis thaliana* was used as a query in a BLAST search against the *Brassica oleracea* genome to find sequences with high homology. The search yielded a single candidate gene with over 70% sequence similarity, which we identified as the probable myrosinase ortholog in broccoli. This candidate gene has a length of 1827 base pairs, with 1647 base pairs forming the coding region.

Phylogenetic Analysis. To further confirm that this gene is the functional ortholog of the myrosinase enzyme encoding gene, a phylogenetic analysis was conducted. Using MEGA (Molecular Evolutionary Genetics Analysis) software, a phylogenetic tree was constructed, including myrosinase gene sequences from *Arabidopsis thaliana.*

Gene Annotation. Using the obtained sequence data, gene annotation tools such as UniProt and Pfam were employed to identify characteristic domains within the myrosinase gene product. Specifically, this included the identification of the glycoside hydrolase family domains, which are critical for enzymatic activity in glucosinolate breakdown.

This bioinformatics approach aims to ensure specificity and precision in the CRISPR knockout experiment. The combined use of BLAST searches, phylogenetic analysis, and domain annotation confirms that the identified gene is functionally aligned with glucosinolate hydrolysis. This targeted approach is critical to achieving the intended reduction in broccoli's bitterness by minimizing glucosinolate breakdown, directly addressing the study's hypothesis. (Figure 1)

Plasmid design

After identifying the TGG1 sequence, the CRISPR Cas9 system was designed and followed the protocol from Plant Genome Editing with the CRISPR System (11), which provides a guide for editing the genome of *Brassica oleracea*. Broccoli is a cultivar of the family (*Brassica oleracea var. italica*). The protocol for plasmid design uses the Golden Gate assembly method. The golden gate assembly technique assembles multiple DNA fragments from different starting plasmids into a single final plasmid. The construction of the final plasmid includes:

- L1P1: kanamycin selection cassette (neomycin phosphotransferase gene (*nptII*), driven by the 35s

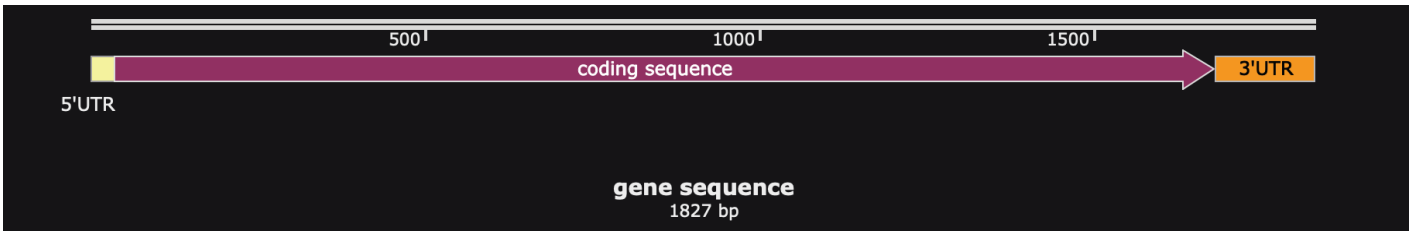

Figure 1. Map of myrosinase enzyme encoding gene gene sequence of broccoli. The 5' UTR is the first 35 bp and the 3' UTR is the last 245 bp. Leaving the other 1647 bp as a coding sequence. The coding sequence encodes protein starting from the start codon and ends at the end codon.

promoter)

- L1P2: Cas9 expression cassette(*Streptococcus pyogenes* Cas9 with the nuclear localization signal, driven by the cassava vein mosaic virus promoter)
- L1P3 and L1P4: guide RNA expression cassettes(both driven by the *Arabidopsis thaliana* U626 promoter)
- L2: accepter plasmid

All the L1 plasmids will construct the T-DNA section of the final plasmid, which is the part being transferred into the plant genome. The L1 plasmid includes the antibiotic for the plant cell (L1P1), the Cas9 gene (L1P2), and two gRNAs (L1P3 and L1P4). The antibiotic resistance gene (*nptII*) allows for the selection of transformed plants on kanamycin containing media during the tissue culture process. The Cas9 gene will direct the plant cell to produce the Cas9 enzyme, while the two distinct gRNAs will guide the Cas9 enzyme to locate and cleave the target DNA sequence. The guide RNA sequence was designed with the help of the CHOPCHOP online CRISPR sequence designer tool to promise a high success rate. CHOPCHOP helped evaluate the gRNA by considering multiple aspects such as self-complementarity, off target scores, on target scores and location in the gene (12). To minimize off target effects, we performed a BLAST search of the potential gRNA sequences against the *Brassica oleracea var. italica* genome to ensure they didn't have significant homology to other important genes. To increase the likelihood of successful gene knockout, the plasmid included two gRNAs, targeting different locations in the myrosinase gene to maximize success rate. The efficiency of the gRNAs was determined by the CHOPCHOP tools scoring system.

These are the two gRNA sequences:

- 5' CTGCTTACCAGATCGAAGGAGGG 3'
- 5' GCGGCTCCATATACCATCCATGG 3'

After finding the suitable knockout sequence, these sequences needed to be inserted into the plasmid. Both sequences need overhangs to facilitate insertion into the plasmid because when we cut the plasmid apart to make it ready for insertion, the enzyme will cut the plasmid in a stick-end way. This means that it doesn't cut at the same place on the two strands, to pair up both strands. So, complementary oligos are required for the successful insertion of the gRNA sequences into the plasmid.

For the L1P3 plasmid, this study used the Bsa1 restriction enzyme for cloning. The generic template of the oligos will be

5'ATTGNNNNNNNNNNNNNNNNN 3'

3' NNNNNNNNNNNNNNNNNNCAAA5'

For the L1P4 plasmid, this study used the Esp31 restriction enzyme, which requires the following overhangs:

5' AGTGCTTGNNNNNNNNNNNNNNNNNNG TTT 3'

3' GAACNNNNNNNNNNNNNNNNNNCAAAA TCT 5'

After inserting the gRNA sequences into the L1P3 and L1P4 plasmids, we digested all L1 plasmids with Bpi1 and ligated them into the L2 acceptor plasmid via Golden Gate assembly to make the final plasmid (Figure 2).

CRISPR-Cas9 Delivery through *Agrobacterium*-Mediated Transformation

The transgene will be introduced into broccoli cells using *Agrobacterium tumefaciens*-mediated transformation. The bacterium carrying this plasmid is will be co-cultivated with broccoli leave tissue, where it transfers the CRISPR construct directly into the plant cells' genomes.

Genotyping

After delivering the plasmid, it is essential to confirm successful gene knockout by sequencing the target site for mutations introduced by the CRISPR-Cas9 system. In this experiment, Polymerase Chain Reaction (PCR) will be used followed by Sanger sequencing to detect mutation

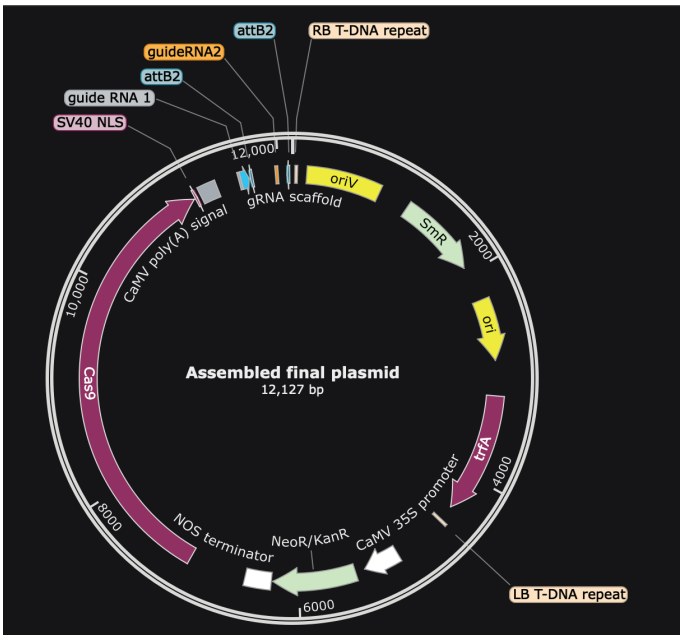

Figure 2. Dual guide vector. The plasmid ready for delivery to the target cells is shown in Figure 2, with a sequence length of 12,127 base pairs.

at the target site. PCR can amplify a large quantity of target sequence, and the amplified product can then be sequenced to confirm if the knockout was successful. The PCR process will require primers to amplify the sequence. The primer design follows a process similar to that of the gRNA design, considering factors like GC content and self-complementarity. When designing primers, it is important to consider GC content, potential self-complementarity, melting temperature, and primer specificity. Two primers that flank both target sites were selected. Two primers were selected to flank both CRISPR target sites, enabling amplification.

Left (forward) primer: 5' CGAAGAGAACAATCCA TTCACA 3'

Right (reverse) primer: 5' CCTACTACCCACAATCT GCCTC 3'

These primers will amplify a region that encompasses the two CRISPR target sites allowing detection of any mutations. After PCR amplification, the product will be purified and sequenced using Sanger sequencing. Afterward, the cells are grown on a selective medium to identify those that have successfully integrated the transgene using the selection marker in the plasmid. These selected cells are regenerated into whole plants and further screened to confirm the intended gene knockout, ultimately leading to a reduction in the bitter-tasting compounds in broccoli.

RESULTS

Following the successful delivery of the plasmid into the broccoli leaf samples using *Agrobacterium tumefaciens* strain AGL1, it is anticipated that individuals sensitive to bitterness in unmodified broccoli would perceive a reduction in bitterness. The anticipated reduction in bitterness is expected because of the absence of the myrosinase enzyme, which is the result of knocking out the myrosinase enzyme encoding gene. Myrosinase enzyme typically hydrolyzes glucosinolates to produce bitter-tasting compounds such as isothiocyanates, isothiocyanates are the main source of bitter taste there for the reduction of isothiocyanates will lead to the reduction of bitter taste. This outcome could be confirmed through a series of biochemical evaluations such as identifying the transformed sequence, the sequence should reveal frameshift mutations or deletion of the myrosinase enzyme encoding gene sequence, indicating the successful gene knockout. The success of the experiment could be also confirmed by the reduction of glucosinolate breakdown products. The final result of reduced bitterness can be determined by recruiting participants who perceive bitterness when consuming broccoli to assess if they perceive reduced bitterness in the modified broccoli compared to the unmodified samples of broccoli.

DISCUSSION

While the primary objective of this study is to reduce the bitter taste of broccoli through the knockout of the myrosinase enzyme-encoding gene, it is essential to acknowledge the potential consequences on brocolli's plant defence system. Myrosinase enzyme plays a critical role in the plant's natural resistance to pests, as it facilitates the breakdown of glucosinolates into defensive compounds that deter insect feeding. By removing this enzyme, we may inadvertently increase the susceptibility of broccoli plants to insect infestations, which could lead to greater agricultural challenges.

This research highlights the trade-off between enhancing consumer preference through reduced bitterness and maintaining the agricultural suitability of broccoli crops by preserving their natural pest resistance. Future studies should investigate ways to balance the pros and cons. Additionally, engineering approaches may

explore modifying other defence pathways to maintain pest resistance in the absence of myrosinase.

CONCLUSION

The successful development of a non-bitter broccoli variant holds significant potential benefits for both consumers and the food industry. A broccoli strain with a milder flavour profile is likely to appeal to a broader audience, increasing its consumption and maximizing its associated health benefits. Moreover, the implications of this research extend beyond broccoli to other members of the Brassicaceae family, where similar modifications could enhance the palatability of these plants. By improving the taste of cruciferous vegetables, we can potentially increase their presence in diets worldwide, fostering greater acceptance of these nutritious foods and contributing to improved public health outcomes. As such, this study serves as a foundation for future research aimed at optimizing flavour while preserving essential plant defences, ultimately promoting sustainable agricultural practices and consumer satisfaction.

CONFLICT OF INTEREST

The author(s) declare that there are no conflicts of interest regarding the publication of this article.

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