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Designing CRISPR-Cas9 Constructs to Target the *SIFSR* Gene in Tomato Varieties Pusa Ruby and UC82 for Extended Shelf Life

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Abstract

This research focuses on the strategic design and implementation of CRISPR-Cas9 constructs to target the Solanum lycopersicum Shelf-Life Food Regulator (SlFSR) gene in two tomato varieties, Pusa Ruby and UC82, to extend post-harvest shelf life. Precise gene editing was done by meticulously selecting target sites within the SIFSR gene and optimizing guide RNA sequences. The CRISPR-Cas9 cassettes were PCR-amplified, cloned into vectors, and ligated to generate the final constructs. Subsequently, these constructs were introduced into both tomato varieties using appropriate delivery methods and transformation protocols. Our findings underscore the importance of precision engineering techniques such as cloning, PCR, and ligation in leveraging genetic editing for agricultural purposes. This research contributes insights into refining CRISPR-Cas9 technology for targeted genetic modification, particularly in enhancing the shelf life of fresh produce, thereby promoting sustainable food practices.

Keyword: CRISPR-Cas9, shelf life, genetic engineering, food security, Tomato

1. Introduction

Tomato (Solanum lycopersicum) is an important crop, second only to potato in global output (Kumar *et al*.,2020). According to FAO (2021), cultivated area and output have increased over the past decade, the area of land used for agriculture has increased from 4.4 to 5 million hectares, and the production of crops has increased from 153 to 181 million tons, respectively. In almost every country on the earth, it is cultivated in the fields, greenhouses, and net houses. Tomatoes are a versatile crop that may be grown for fresh markets or processing (Bhatia *et al*., 2003; Gatahi *et al*., 2020). Asia accounted for over 62 percent of regional production, followed by the Americas (13.2 percent), Europe (12.6 percent), and Africa (12 Percent). China (mainland) produces 62.8 million tons of tomatoes, followed by India (19 million tons), Turkey (12.8 million tons), the United States (10.9 million tons), and Egypt (6.8 million tons).
Tomatoes belong to the Solanaceae family, which has approximately 3000 different species. According to Liu *et al*. (2022), the tomato is a model plant species since it is both an economically significant crop and a model plant species owing to its diploid, relatively compact, and newly sequenced genome, including its rich genetic and genomic assets. Tomatoes are good for human health since they reduce the risk of cancer, osteoporosis, and cardiovascular disease (Melilli *et al*., 2007; Ali *et al*., 2020). In everyday diets, tomatoes contain high levels of carotenoids (particularly carotene and lycopene), vitamin C (Brezeanu *et al*., 2020), phenolic

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components, and minor amounts of vitamin E (Causse et al., 2016). Tomatoes are also high in secondary metabolites like phosphate, potassium, flavonoids, and chlorophyll, which benefit human health (Ali *et al*., 2020).

Tomatoes are becoming increasingly popular as a result of their high nutritional content. Several biotic and abiotic stresses affect its production, including diseases caused by fungi, bacteria, viruses, and nematodes. Extreme temperatures, drought, salt, and vulnerability to insect and pest infestations are only a few environmental elements that negatively influence crop growth and productivity (Renaud, 2014; El-Garhy *et al*., 2020). Disease outbreaks are a well-known occurrence that lowers agricultural output and raises production expenses. According to Fentik (2017), enhancing this crop is critical to addressing tomato production constraints.

To overcome these challenges and improve tomato production in Nigeria, advanced biotechnological approaches, such as genetic engineering, offer promising solutions. Genetic engineering techniques allow for the precise manipulation of plant genomes, introducing desirable traits that can address specific agricultural challenges (Foolad, 2007; Zahir *et al*., 2018). The CRISPR/Cas-mediated genome editing technique represents a transformative advancement in plant genome modification. (Wang 2022) The effectiveness of this method primarily depends on carefully selecting a specific vector and its components. A typical plant-specific CRISPR/Cas vector comprises a Cas gene, a target-specific guide RNA (gRNA), a leader sequence, a selectable marker gene, appropriate promoters, and additional elements, as noted by Alok (2021).

2. Materials and Methods

2.1 CRISPR/Cas9 Binary Vector

The CRISPR/Cas9 binary vector pRGEB31 was procured from Addgene, the culture was obtained as stab culture. The culture was revived on LB agar medium supplemented with 50.0mg/L Kanamycin. 15% glycerol stocks were prepared and stored in deep freezers for long-term storage.

2.2 Bacterial Strains

E. coli strain DH5α and Agrobacterium tumefaciens strain LB 4404 were used in the study. The bacterial strains used in the study were obtained from the ICGEB-International Centre for Genetic Engineering and Biotechnology, New Delhi, India. The cultures were maintained on LB agar plates with suitable antibiotics and glycerol stocks.

2.3 Retrieval of Gene Sequence Data of SIFSR Gene

The sequence information of the tomato gene SlFSR Solyc07g052960, identified as a direct target of RIN, was obtained from Photozome 13 (https://phytozome-next.jgi.doe.gov/).

2.4 Designing of Guide RNAs (gRNAs)

The guide RNAs (gRNAs) 20 bp long for editing the SlFSR gene were designed using the free online Crispor Bioinformatic tool (http://crispor.org/). RNAs were selected based on their on-score values, GC content, number of off-target sites, and location in the genome.

2.5 Designing of Primers

Primers for amplifying partial gene sequences of the SlFSR gene were manually designed and analyzed for their features using the "Oligoecalculator" tool (Sigma-Aldrich).

2.6 Confirmation of SlFSR Gene in Tomato Genome

The tomato genomic DNA was isolated from the leaf of Solanum lycopersicum Pusa Ruby, and UC82 tomato Genotypes were obtained from the International Center for Genetic Engineering and Biotechnology (ICGEB) in New Delhi, India, following the CTAB method [Rogers et al. 1994]. The target region for CRISPR/Cas9-mediated editing of the SlFSR gene was amplified using polymerase chain reaction (PCR)

using gene-specific flanking primers. The PCR product was checked on 1 % gel, and sequences were confirmed.

2.6.1 Quantification and Quality Check of DNA

The DNA concentration was determined spectrophotometrically at 280 nm (A280) absorption using NanoDrop1000 (Thermo Scientific). The purity of DNA was assessed by estimating the absorbance ratio at A260/A280 and A260/A230, respectively. The quality of the extracted DNA was also evaluated by electrophoresis separation for all DNA samples on 0.8% agarose gel stained with ethidium bromide (1 µg/ml).

2.6.2 Agarose gel electrophoresis

Nucleic acids, including genomic DNA, plasmids, or PCR products, were loaded on the agarose gel (concentrations ranging from 0.8% to 1.2% of agarose as per requirement) using a 6X DNA loading dye (Thermo Fisher Scientific) and containing 5 µg/ml of ethidium bromide (EtBr). A 1kb ladder from Thermo Scientific was also loaded alongside the samples for reference. The process of gel electrophoresis was performed in a buffer of 1X TAE, comprising 40 mM Tris base, 20 mM acetic acid, 1 mM EDTA, and a pH of 8.0. The electrophoresis was conducted at a voltage of 70V/cm using electrophoresis units from Bio-Rad, USA. Following the electrophoresis, the DNA that had been separated was visualized using a gel documentation system (Alpha Imager, USA).

3. Result and Discussion

3.1 Designing of Guide RNAs (gRNAs)

The full-length sequence of the tomato SlFSR gene (accession no. Solyc7g052960) was retrieved from a tomato genome database. Using CRISPOR, two single guides were designed according to Liang et al. (2016). CRISPOR stands out for its user-friendly interface and comprehensive database, which incorporates genomic data from various organisms, including plants (Concordet et al, 2018) (https://omicstutorials.com/top-10-free-open-source-genome-analysis-tools-for-researchers/). The free tool facilitates the identification of the most effective gRNA sequences by evaluating factors such as on-target efficiency and off-target potentials. This evaluation is crucial, as it directly impacts the specificity with which the CRISPR-Cas9 system can target and modify the desired genomic region without affecting other parts of the genome.

3.2 Amplification and cloning of FSR into pRGEB31

Targeting the SlFSR gene, the sgRNA insert was amplified via Polymerase Chain Reaction (PCR) Fig (c), employing precisely designed forward and reverse primers. This critical step ensured the specificity and efficiency of the sgRNA, which are paramount for the precision of the CRISPR-Cas9 system's editing capabilities (Feng *et al*., 2021) (Aljabali *et al*., 2024).

Following PCR amplification, electrophoresis analysis confirmed the successful amplification of the sgRNA insert, as evidenced by a distinct band corresponding to the expected size. This result underscored the precision of our primer design and the effectiveness of the PCR conditions employed, aligning with established protocols for sgRNA amplification (Liang *et al*., 2016). The amplified sgRNA product was then ligated and cloned into the pRGEB31 vector, a crucial step for creating the tRNA-based multiplex sgRNA expression system.

Importantly, the pRGEB31 vector employs the U6 promoter to drive the expression of the inserted sgRNA scaffold. The U6 promoter is recognized for its efficiency in transcribing small nuclear RNA molecules, including sgRNAs, across a variety of eukaryotic cells (Long *et al*., 2018; Wang *et al*., 2021). This feature of the pRGEB31 vector ensures robust sgRNA expression, optimizing the CRISPR-Cas9 mediated genome editing process. The SIFSR pRGEB31 - Cas 9_U6 plasmid was digested with the Bsal restriction enzyme to validate gene insertion via electrophoresis Fig 1(d).

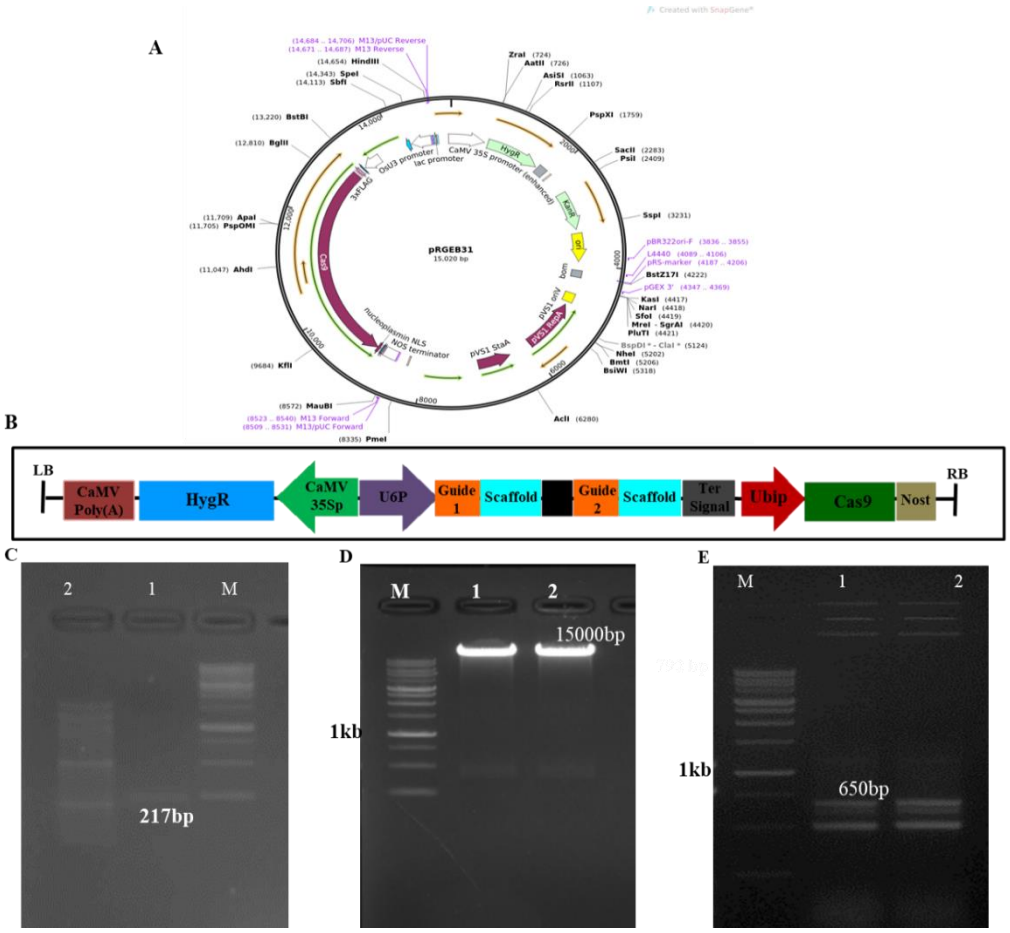

Fig 1: Cloning of FSR in pRGEB31 for plant transformation: (a)Schematic representation of pRGEB31 Vector (b) Pictorial representation of the construct containing the FSR gene. The insert and digested vector before ligation are depicted in the gel images (c) and (d). In Figure (e), the gel image illustrates the restriction digestion of plasmid extracted from colony PCR-positive clones,

4. Conclusion (Follow the style used in this Section: 12 fonts)

Advancements in genome editing technologies present exciting opportunities for precise and targeted modifications of the plant genome. Furthermore, characterizing CRISPR-edited tomato plants under field conditions is essential to assess their agronomic performance, shelf life, and resilience to biotic and abiotic stresses. Field trials provide valuable insights into the real-world behavior of genetically modified crops, informing future breeding strategies and deployment practices. Understanding the complex interplay between plants and Agrobacterium during infection can also inform plant breeding efforts to control crown gall disease. Leveraging insights from studies on plant immune responses and signal transduction pathways could enable the development of disease-resistant tomato varieties through targeted genetic modifications.

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