doi 10.18699/PlantGen-2025-278

In vitro CRISPR-Cas9 cleavage assay for targeted editing of *TGMS5* gene in wheat

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Motivation and aim: Wheat is one of the most important cereal crops grown worldwide and plays a vital role in ensuring global food security. However, recent wheat yield gains have lagged behind other major crops such as rice and maize due to climate variability and rising demand. The development of hybrid wheat varieties is essential for improving yield potential and ensuring global food security. Thermo-sensitive genic male sterility (*TGMS*) is a promising tool for developing high-yielding hybrid wheat. However, the complexity of the wheat genome and limitations in transformation efficiency make precise genome editing difficult. CRISPR/Cas9 genome editing presents a revolutionary alternative, allowing targeted disruption of *TGMS5* gene male-sterile lines. However, the efficiency of CRISPR editing in wheat remains constrained by its polyploid genome, low transformation rates, and the risk of off-target effects.
The aim of this study was to design and evaluate CRISPR-Cas9 guide RNAs (gRNAs) targeting the *TGMS5* gene using an *in vitro* cleavage assay. By screening gRNA candidates for cleavage efficiency and specificity prior to plant transformation, we aim to improve the accuracy and success rate of gene editing in wheat. This approach will support future efforts in generating stable *TGMS* lines and advancing hybrid wheat breeding technologies.
Materials and methods: Genomic DNA was extracted from wheat leaves and used to amplify target regions of the *TGMS5* gene via PCR. Ten guide RNAs (sgRNAs) were designed using Wheat CRISPR bioinformatics tool (https://crispr.bioinfo.nrc.ca/WheatCrispr/), aiming to identify highly efficient and homoeoallele-specific candidates for wheat genome editing.
Results: An *in vitro* assay was conducted to test the cleavage efficiency of selected gRNAs targeting the *TGMS5* gene. It included an *in vitro* gRNA transcription, gRNA and Cas9 assembly to RNP complexes and incubation with PCR-amplified *TGMS5* fragments. Primers were designed to generate amplicons with distinct fragment sizes, enabling clear visualization of cleavage events via agarose gel electrophoresis. All the tested gRNAs showed clear and consistent cleavage activity, with exhibiting the highest efficiency. These results closely matched *in silico* predictions generated using the Wheat CRISPR tool. The validated gRNAs demonstrate both high efficiency and specificity, making them strong candidates for CRISPR/Cas9-mediated genome editing in wheat breeding and functional genomics.
Conclusions: This study we have successfully combined *in silico* analysis and *in vitro* validation to identify highly efficient and specific gRNAs targeting the *TGMS5* gene in wheat. By using a CRISPR/Cas9 *in vitro* cleavage assay, we were able to pre-screen and optimize guide RNAs prior to plant transformation, minimizing the risk of off-target effects and low editing events efficiency. The validated gRNAs provide reliable tools for precise genome editing and lay a strong foundation for developing thermo-sensitive male sterile lines in wheat. These findings contribute to advancing CRISPR-based strategies in wheat breeding, with potential to accelerate the development of high-yielding, climate-resilient hybrid varieties.
Funding: The study is supported by No. 1125_Sk Foundation grant.