

CONSTRUCTION OF CRISPR/CAS9 VECTOR FOR SILENCING *CIF1* GENE OF TOMATO

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ARTICLE INFO	ABSTRACT
Received: 11/8/2021	Tomato (<i>Solanum lycopersicum</i>) is a nutrient-dense food that contains various secondary compounds, as well as great health benefits. The sugar content of tomato fruit is partly controlled through the regulation and breakdown of sucrose in fruit set and development. Cell wall invertase (CWI) hydrolyzes sucrose into monosaccharides and transports it into the cytoplasm, which means the sugar content of tomato is regulated by CWI. Meanwhile, since this gene repression is induced by a product of the <i>CIF1</i> gene, inactivation of the <i>CIF1</i> gene may enhance the sugar synthesis in tomatoes. Currently, CRISPR/Cas9 system is a state-of-the-art technology, has wide applications and high precision in gene editing. In this study, suitable gRNAs for the <i>CIF1</i> gene were designed to build expression constructs. This expression system in the pRGE31-CIF1G2 plasmid was introduced into DH10B <i>Escherichia coli</i> strain. Afterwards, the vector carrying this expression system was successfully transferred into the EHA105 <i>Agrobacterium tumefaciens</i> strain. Further, <i>A. tumefaciens</i> lines containing the vector pRGE31-CIF1G2 can be used to generate desired traits in gene-edited Tiny-Tim tomato lines.
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THIẾT KẾ VECTOR CRISPR/CAS9 ĐỊNH HƯỚNG BẮT HOẠT GEN *CIF1* Ở CÀ CHUA

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THÔNG TIN BÀI BÁO	TÓM TẮT
Ngày nhận bài: 11/8/2021	Quả cà chua (<i>Solanum lycopersicum</i>) là một loại thực phẩm giàu chất dinh dưỡng có chứa nhiều hợp chất thứ cấp khác nhau, cũng như nhiều lợi ích đối với sức khỏe. Hàm lượng đường trong quả cà chua được kiểm soát một phần thông qua việc điều hòa và phân hủy lượng đường sucrose trong quá trình đậu trái và phát triển quả. Enzyme phân hủy vách tế bào (CWI) thủy phân sucrose thành monosaccharide và vận chuyển vào tế bào chất, đồng nghĩa với hàm lượng đường trong cà chua được điều chỉnh bởi CWI. Trong khi đó, do sự ức chế gen này được gây ra bởi một sản phẩm của gen <i>CIF1</i> , việc bất hoạt gen <i>CIF1</i> có thể tăng cường tổng hợp đường trong cà chua. Hiện nay, hệ thống CRISPR/Cas9 là công nghệ hiện đại, có ứng dụng rộng rãi và độ chính xác cao trong chỉnh sửa gen. Trong nghiên cứu này, các gRNA thích hợp cho gen <i>CIF1</i> được thiết kế để xây dựng các cấu trúc biểu hiện. Hệ thống biểu hiện này trong plasmid pRGE31-CIF1G2 đã được đưa vào chủng <i>Escherichia coli</i> DH10B. Sau đó, vector mang hệ thống biểu hiện này đã được chuyển thành công vào chủng vi khuẩn <i>Agrobacterium tumefaciens</i> EHA105. Các dòng <i>A. tumefaciens</i> chứa vector pRGE31-CIF1G2 có thể được sử dụng để tạo các dòng cà chua Tiny-Tim chỉnh sửa gen mang các đặc điểm mong muốn.
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TỪ KHÓA	
Cà chua	
CRISPR/Cas9	
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Vector chuyển gen	

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1. Introduction

Tomato (*Solanum lycopersicum*) is a plant belonging to the *Solanaceae* family, known for its high nutritional value to humans. It is also one of the most economically valuable food, currently grown and consumed all over the world. Tomatoes are grown worldwide for local use or as an export crop, with increasing commercial value annually. In 2014, the global cultivated area was recorded at 5 million hectares, with production amounting to 171 million tons. Therefore, there has been the number of studies on tomatoes conducted for many decades to increase the understanding of tomato, in order to improve fruit yield and quality.

During plant growth, hormones play an important role in regulating appropriate development. Through each stage of growth and development, plant physiology is regulated by various phytohormones, such as auxin, gibberellin, cytokinin, abscisic acid, etc. Besides, the sugar content in fruit, which accounts for roughly 50% of the dry weight, plays many important roles in the fruit formation and development, as well as the sweetness of the ripe fruit. The sugar content in the fruit increases gradually from the mature green stage to the red ripe stage [1], is mainly glucose and fructose in equal proportions. The sucrose (disaccharide) content is negligible (less than 0.1%), which is transported from the leaves to the fruit, thus the sweetness of ripe tomatoes is chiefly counted on the level of monosaccharides [2], [3]. The sugar content of tomatoes is regulated by two types of mosaic acid invertase on the cell wall (Cell wall invertase - CWI), which hydrolyzes sucrose into glucose and fructose, then transports into cytoplasm vacuole invertase (Vacuole invertase-VI) contribute to maintain the ratio of sucrose/hexose in the cytoplasm [4]. Invertases are inhibited by the invertase inhibitors (INVINHs) through the post-translation regulatory mechanism, that may interact to create inactivated complexes. The expression of INVINHs is regulated by abscisic acid and is directly related to the tolerance responses with cold weather of tomatoes [5], [6]. Presently, 3 genes encode for INVINHs were isolated, including a gene encoding for VI inhibitory protein (*VIF*) and other 2 genes coding for CWI inhibiting proteins (*CIF1* and *CIF2*). In tomatoes, *VIF* expression is inhibited by a synthesized ripening inhibitor (RIN) transcription regulator, while *CIF1* and *CIF2* are enhanced by this factor [4]. *CIFs*, which belong to the family of proteins involved in pectin methylesterase inhibitor (PMEI-RP, Pfam: 04043), are determined based on the activities of invertase acids. *CIFs* regulate the efficiency of the invertase enzyme and signal flow of glucose down *in vivo*, thereby increasing sucrose content [4]. Therefore, inactivation of the *CIF1* gene would theoretically increase the expression level of the CWI gene, by that improving the sugar content of tomatoes. So far, based upon our understanding, we have found no published report on *CIF1* gene inactivation by CRISPR/Cas9 technology. Besides previous gene editing methods such as ZFNs or TALENs, CRISPR/Cas9 is a state-of-the-art technology that provides outstanding precision. With the goal of creating a gene-edited Tiny-Tim tomato variety to enhance fruit quality through sugar content, in this study, the CRISPR/Cas9 method was applied to modify the *CIF1* gene in tomato in order to inactivate this gene, which means improving sugar levels through downstream reactions. The recombinant DNA will then be transferred into *Agrobacterium tumefaciens* and available for plant transformation.

2. Materials and method

2.1. Materials

The transgenic vector pRGE31 (15.037-bp-length, Figure 1) was purchased from Addgene (MA, USA). This binary vector is utilized to generate stable transgenic plants by agrobacterium-mediated transformation. Young leaf samples of Tiny-Tim variety and two bacteria strains (DH10B *E. coli* and AHA105 *A. tumefaciens*) were provided by Genome Biodiversity Laboratory, Institute of Genome Research.

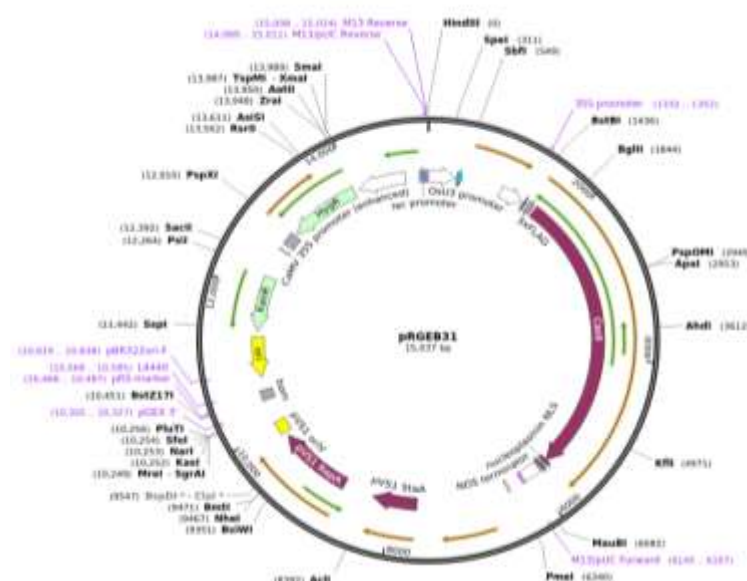


Figure 1. Schematic structure of pRGE31 vector

Primer sequences are designed based on the *CIF1* sequence (Accession number: NC_015449.3) and presented in table 1. Sequence construction and bioinformatics processing were performed using Primer-Blast and BioEdit tools.

Table 1. List of primers

Number	Primer	Nucleotide sequence (5'-3')	Length (bp)	Purpose
1	Sol200E1-F	CCCACCGAAAACACAAAGCA	20	Amplification of <i>CIF1</i> 's exon 1 region
2	Sol200E1-R	GCAACCAGTAAGATAGGGTTCGAA	23	
3	OsU3-R	CTGGAGATTATTGCTCGGGTAGA	22	Colony screening
4	OsU3-F	GAACACTGGGTACGTTGGAAC	22	

2.2. Methods

2.2.1. Total DNA extraction

DNA extraction was performed according to the CTAB method [7] with few modifications to adjust the samples. In short, total DNA was extracted by crushing the young leaves in liquid N₂, centrifuged at high rpm to collect suspension and precipitated with CH₃COONa and 100% ethanol. The precipitation was washed with 70% ethanol and RNase to remove any undesired impurities, protein or RNA. DNA quality was confirmed by electrophoresis in 1% agarose gel.

2.2.2. Specific gRNA designing for *CIF1* gene

SlCIF1 sequence was referenced from the NCBI database (NC_015449.3), in order to specifically designed the corresponding primer pair (Sol200E1-F/R, table 1) to amplify the Exon1 region. The desired fragment was then sequenced by the Sanger method. Appropriate gRNA sequences were designed based on the obtained sequence and the CRISPR-P version 2.0 web-tool (<http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/CRISPR>). The outcome with the most suitable index was selected for synthesis, in which the forward primer contains a 5'-GGCA-3' adapter (gRNA-spacer begins with A) or 5'-GGC-3' (gRNA- padding begins with G / C / T), while the reverse primer contains a 5'-AAAC-3' adapter.

2.2.3. gRNA ligating into pRGEB31 vectors

Denaturation and renaturation reaction was performed at 95°C for 5 minutes to create a double stranded strand of gRNA, then cooled down to 25°C for 30 minutes. The cut-ligated reaction to create recombinant vectors was performed with 1 µl of each reaction complementary pairing of gRNA2, 100 ng of pRGEB31 vector, 3 µl T4 ligase 10X, 3 µl BSA 1 mg/ml, 0.5 µl BsaI (Thermal Scientific), 1 µl T4 ligase. Thermal cycling started at 37°C for 5 minutes, then repeated 40 cycles (20°C:5 minutes, 37°C:5 minutes), extended 10 minutes at 50°C and finished after next 10 minutes at 80°C.

2.2.4. Plasmid transformation into *E. coli* by heat shock

To generate the recombinant vector carrying the desired gene fragment in DH10B *E. coli*, the vector product was mixed with competent cells (1:10 volume ratio). After 30 minutes of incubation, the thermal shock was performed at 42°C for 60 seconds, followed by rapid cooling in ice for 5 minutes. The mixture was supplemented with liquid LB, incubated for 1 hour while shaking at 200 rpm. Finally, bacteria were cultured on Petri dishes, supplemented with kanamycin (50 µg/ml) and incubated overnight at 37°C.

2.2.5. Colony screening

By performing PCR reaction, the *CIF1-G1* gene was checked by the OSU3-R, CIF1G1-F primer and the *CIF1-G2* gene was checked by the OSU3-R, CIF1G2-F primer to ensure that the vector carries the correct insertion. The optimal reaction composition was similar to that of *SICIF1* gene amplification, along with 32 cycles and annealing temperature in the range of 51-54°C. Products were confirmed by electrophoresis.

2.2.6. Plasmid extraction

Colonies had positive PCR result were grown in liquid LB containing 50 ng/ml kanamycin for plasmid extraction. The cultured cells were centrifuged at 13,000 rpm for 1 minute to discard supernatant, supplemented with 150 µl Sol I, 200 µl Sol II and 150 µl Sol III; then instantly cooled on ice for 5 minutes. Next, DNA was centrifuged at 12,000 rpm for 15 minutes, precipitated with 100% ethanol and incubate at -80°C. After one hour of incubation, the precipitate was washed with 300 µl of 70% ethanol, dried and stored at -20°C. The results were confirmed by electrophoresis.

2.2.7. Plasmid transformation into *A. tumefaciens* cells by electroporation

EHA105 *A. tumefaciens* strain was selected as a plant transgenic vector to contain recombinant genes. Plasmids were transferred into the competent cells by the electroporation method. The recombinant product was mixed with EHA105 cells in 1:10 ratio and transferred to curvet tube. Cells were electroshocked at 2500V, 200 Ω, 25 µF, then supplemented with liquid LB and recovery culture, incubated for 1 hour while shaking at 200 rpm and 28°C. Cells were then inoculated in a petri dish containing kanamycin (50 µg/ml), incubated overnight at 28 °C.

3. Results and discussion

3.1. Total DNA extraction of *Tiny-Tim* tomato

Total DNA of leaf samples was obtained and examined for quality by electrophoresis on 1% agarose gel. The results in Figure 2 show sharp and mostly unbroken DNA bands, and the RNA had been generally removed. OD value (260/280 nm) of DNA product was 1.875, which means that the quality of the DNA was sufficient for the downstream experiment.

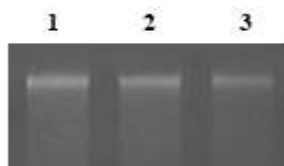


Figure 2. Total DNA of leaf samples

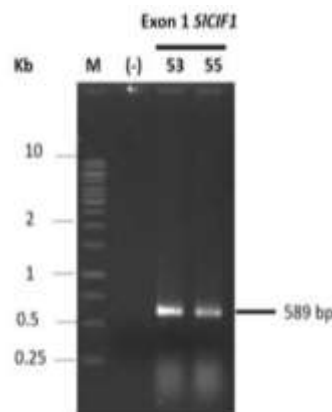
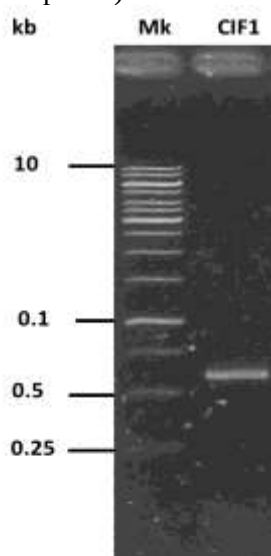


Figure 3. PCR optimization for of exon 1 region; 53, 55: Optimal temperature for exon 1 amplification; M: 1kb DNA ladder

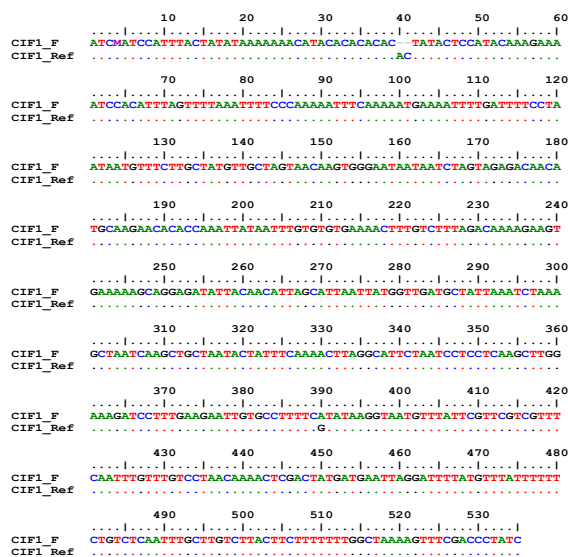
3.2. Amplifying and sequencing for *SICIF1* exon 1 region

Exon 1 region in the *SICIF1* gene was selected for amplification, using the specific primer pairs Sol200E1-F and Sol200E1-R. This primer pair has a theoretical annealing temperature ranging from 53 to 55°C. To obtain the best results, PCR reactions were performed at both temperatures. Electrophoresis image (Figure 3) shows that both temperatures result in a single 600-bp-length band (theoretical length is 589 bp). This result also once again confirms the specificity of primer designed for exon1 region.

After obtaining the desired exon 1 sequence, the PCR product was purified to remove any excess substances that may interfere with sequencing quality. The results in Figure 4A demonstrate that the DNA band was more clear and sharper than the PCR product. Exon 1 region was further sequenced by the Sanger method. After removing the noise peak at both ends, a 535-bp gene fragment (CIF_F) was obtained with high peak quality. The obtained sequences were aligned with its reference from the Genbank database (CIF1_Ref), showing that there were 3 differences (Figure 4B), including 2 indels (AC) at positions 40-41 and 1 change at position 390 (G /A). This sequence was used to design the appropriate gRNA (which did not contain any mutation points).



A



B

Figure 4. Exon 1 regions purified and sequencing result
(A) Purified products (B) Exon 1 alignment

3.3. Specific-designed gRNA for *CIF1* gene

To generate frameshift mutations on the *SICIF1* gene by CRISPR/Cas9 method, it is required to design a gRNA sequence suitable for the target gene. gRNA plays a key role in recognizing the target DNA containing the complementary sequence, and the presence of the PAM (Protospacer-Adjacent Motif) sequence which is critical for the specific recognition and binding process of the Cas9 enzyme into the target gene. gRNAs were designed by CRISPR-P version 2.0 web-tool based on sequenced exon 1 region. For choosing gRNAs, the priorities are significant on-target value and none of mismatching with CDS region of other genes in the tomato genome. Besides, the selected sequence has to follow criteria: GC% content is between 40-60%, priming temperature is between 50-65°C, no secondary structures with large dissociated energy, and also specified to the target gene in the tomato genome.

Table 2. gRNA sequences

No.	Name	Nucleotide sequence (5'-3')	Melting temperature (T _m)	%GC	Length (bp)
1	CIF1-G1-F	GGCACCTATGTTGCTAGTAACAAGT	55	41.7	24
	CIF1-G1-R	AAACACTTGTTACTAGCAACATAG	51.2	33.3	24
2	CIF1-G2-F	GGCACTAATACTATTTCAAACCTT	49.5	29.2	24
	CIF1-G2-R	AAACAAGTTTGTGAATAGTATTAG	45.7	20.8	24

After designing with the web tool and evaluating the parameters, 2 suitable gRNA pairs were selected (Table 2). These gRNAs are in the open reading frame of exon 1 on the *SICIF1* gene. Both of these pairs had high on-target indexes (0.61 and 0.45, respectively) and were specific to the target gene of the study. Therefore, they were synthesized for further experiments.

3.4. Transformation of gRNA-CRISPR Cas9 vector into *E. coli* cells

After transferred gRNAs into the pRGEB31 vector, the recombinant vectors were transformed into *E. coli* cells. A competent DH10B line was chosen to carry the vectors. The *E. coli* cells were then inoculated on Petri dishes containing LB medium and kanamycin. After one day, a number of small, round-shaped colonies were observed. However, the cut-ligated efficiency may not up to 100%, hence confirmation by PCR was necessary.

3.4.1. Colonies selection by PCR screening

To select successfully transformed colonies, PCR screening was conducted. The gRNA segment, which had been inserted into the pRGEB31 vector, was checked by the gRNA1 (CIF1G1-F) or gRNA2 (CIF1G2-F) gRNA oligo 1 as forward primer and the OsU3-R1 reverse primer. The insert-carrying structures were named pRGEB31-CIF1G1 and pRGEB31-CIF1G2. The electrophoresis results (Figure 5) showed that there were 3 colonies pRGEB31-CIF1G1 (3, 4, 5), and 4 colonies pRGEB31-CIF1G2 (1, 2, 4, 5) that had approximate length as expected (230 bp). From these results, these 7 colonies were sequenced to confirm the presence of the gene of interest.

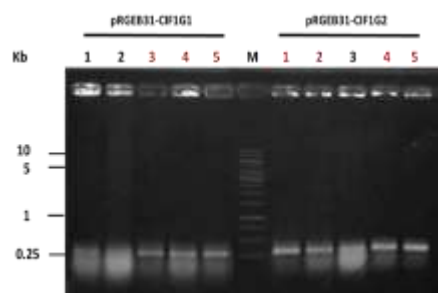


Figure 5. PCR screening result

(A) Colonies carry pRGEB31-CIF1G1 structure (B) Colonies carry pRGEB31-CIF1G2 structure M: 1kb DNA ladder; (-) negative control (without DNA template).

3.4.2. CRISPR/Cas9-CIF1 structure sequencing

Selected colonies were verified by Sanger sequencing for ensuring accuracy with primer OsU3-F. Firstly, 3 colonies carrying pRGE31- CIF1G1 structure (number 3, 4, 5) were sequenced. However, the sequencing results showed that the gRNA fragment was not exactly as designed, which means the pRGE31- CIF1G1 vector was no longer used for any other analyses. Similarly, 4 colonies bearing the pRGE31- CIF1G2 structure (numbers 1, 2, 4, 5) were also sequenced with the forward primers (Figure 6). The result indicated that 3/4 of those (numbers 1, 2, 5) carry the exact insert as designed. Despite the positive PCR results as well as the expected length, the post-sequencing result of #4 was not as accurate as of the designed insert and was discarded.

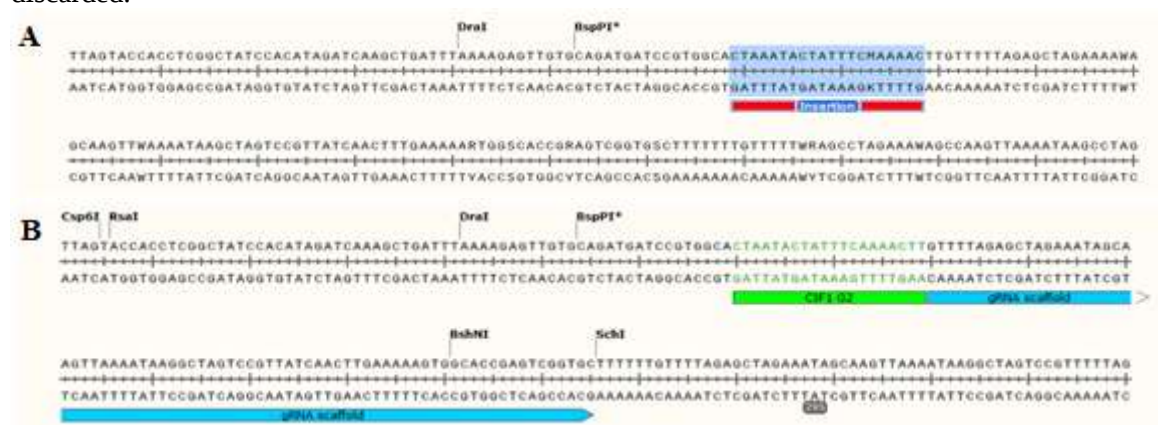


Figure 6. Sequencing gRNA on pRGE31-CIF1G2 vector number 1, 2, 4, 5.

(A) Sequencing gRNA on pRGE31- CIF1G2 vector number 4 (B) Sequencing gRNA on pRGE31- CIF1G2 vector number 1, 2, 5

3.4.3. Transformation gRNA-CRISPR/Cas9 structure into of *A. tumefaciens* cells

The screened pRGE31-CIF1G2 vector was transferred into the EHA105 *A. tumefaciens* line by the electroporation method. Bacterial cells were supplemented with 950 μ l liquid LB and cultured for 1 hour, 37°C; then spread on an agar plate containing kanamycin (50 μ g/ μ l) and cultured overnight at 28°C.

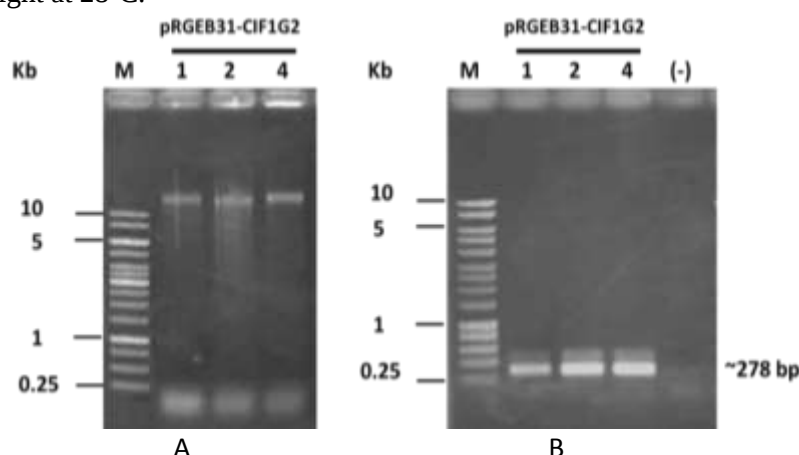


Figure 7. Examined the presence of pRGE31-CIF1G2 structure on *A. tumefaciens* cells (A) Plasmid extraction (B) PCR screening; M: 1kb DNA ladder; (-): negative control

After screening with suitable antibiotic, the results of the transformation of the pRGEB31-CIF1G2 vector into EHA105 strain obtained 4 colonies, however, colony number 3 was unsuccessfully harvested after liquid-cultured phase. Plasmid extraction was performed, resulting in quite clear bands. The plasmid bands were greater than the highest band of the DNA ladder (10 kb), which is consistent with the determined size of the recombinant vector (about 15 kb). PCR was performed for 3 colonies, using OsU3-R and gRNA2 as a primer. Figure 7B presents 3 clear bands corresponding to each plasmid, approximately 300 bp in length as expected. This result confirmed the successful transformation of the pRGEB31-G2 plasmid into the EHA105 *A. tumefaciens* strain.

These are potential plant transgenic vectors for *SICIF1* inactivation in the Tiny-Tim cultivar. Physiological processes in plants are quite complicated, involving many genes, phytohormones, affecting different properties in fruit such as color, flavor, sweetness, etc. In tomato, CWI functions to hydrolyze the disaccharide sucrose to glucose and fructose. Overall, this ensures a stable balance in sugar content, which affects many aspects of plant physiology, such as sugar signaling, fruit sweetness or apoplastic carbon nutrients. On the other hand, CWI activity is tightly controlled by inhibitors at the post-translational level, including the presence of the *CIF1* gene. Research on the mechanism of action and the ability to improve seed germination, fruit quality based on *CIF1* inactivation has been investigated in a number of publications. In Arabidopsis, the loss-of-function mutation in the *CIF1* gene resulted in faster seed germination as well as a significant increase in hexose content, and a significant increase in CWI activity both *in vivo* and *in planta* [8] This shows that post-translational modulation of CWI by *CIF1* positively affects seed germination and development. Another study on tomatoes also showed that enhancing CWI activity through inactivation of *SICIF1* could improve seed weight and fruit hexose content in tomato [6]. A later study by Zhang in 2018 [9] on tomato showed that *CIF1* affects the post-translational regulation of CWI, contributing to earlier flowering; at the same time, this interaction regulates fruit development and tomato fruit quality. Thereby, it shows that the inactivation of the *CIF1* gene has favorable effects on some properties in tomato. Here, we have generated the structure pRGEB31-CIF1G2 by CRISPR/Cas9 method and successfully transferred it into the EHA105 *A. tumefaciens* strain. This advanced technology has also been successfully used in gene editing and inactivation for many different species, but this is the first time it has been applied to the *SICIF1* gene. Theoretically, transgenic plants carrying this construct could reduce the expression level of the corresponding protein, which means enhanced CWI activity compared with the wild-type phenotype. Creating this gene-inactivated construct offers the potential not only to obtain tomato plants with beneficial properties (early seed germination, flowering time, improved fruit quality, etc.), but also to generate gene-edited food varieties that are acceptable (unlike genetically modified foods, which face many risks and are rarely accepted for human consumption).

4. Conclusion

Sequencing the exon1 region of the *SICIF1* gene of Tiny-Tim tomatoes, the results showed that there were 03 distinct nucleotides compared to the reference sequence on the database. The CIF-G2 structure was successfully inserted into the pRGEB31 vector as designed. Also, the *E. coli* DH10B and *A. tumefaciens* EHA105 bacteria strains were genetically engineered which containing the recombinant vector pRGEB31-CIF1G2. The *A. tumefaciens* strain is the available source for plant transformation, promising to create a gene-edited Tiny-Tim tomato variety with beneficial traits as desired.

REFERENCES

- [1] J. N. Davies and R. J. Kempton, "Changes in the individual sugars of tomato fruit during ripening," *Science of Food and Agriculture*, vol. 26, pp. 1103-1110, 1975.

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- [2] C. Davies and S. P. Robinson, "Sugar accumulation in grape berries (cloning of two putative vacuolar invertase cDNAs and their expression in grapevine tissues)," *Plant Physiology*, vol. 111, no. 1, pp. 275-283, 1996.
 - [3] J. N. Davies and G. E. Hobson, "The constituents of tomato fruit--the influence of environment, nutrition, and genotype," *Crit. Rev. Food Sci. Nutr.*, vol. 15, pp. 208-280, 1981.
 - [4] G. Qin, Z. Zhu, W. Wang, J. Cai, Y. Chen, L. Li, and S. Tian, "A tomato vacuolar invertase inhibitor mediates sucrose metabolism and influences fruit ripening," *Plant Physiol.*, vol. 172, no. 3, pp. 1596-1611, 2016.
 - [5] T. Rausch, S. Greiner, and M. LinkIn, "Arabidopsis thaliana, the invertase inhibitors AtC/VIF1 and 2 exhibit distinct target enzyme specificities and expression profiles," *FEBS Letter*, vol. 573, pp. 105-109, 2004.
 - [6] Y. Jin, D. Ni, and Y. Ruan, "Posttranslational elevation of cell wall invertase activity by silencing its inhibitor in tomato delays leaf senescence and increases seed weight and fruit hexose level," *Plant Cell*, vol. 21, no. 7, pp. 2072-89, 2009.
 - [7] J. J. Doyle and J. L. Doyle, "Isolation of plant DNA from fresh tissue," *Focus*, vol. 12, no. 1, pp. 13-15, 1990.
 - [8] T. Su, S. Wolf, M. Han, H. Zhao, H. Wei, S. Greiner, and T. Rausch, "Reassessment of an Arabidopsis cell wall invertase inhibitor AtCIF1 reveals its role in seed germination and early seedling growth," *Plant Mol. Biol.*, vol. 90, no. 1-2, pp. 137-155, 2016.
 - [9] N. Zhang, J. Jiang, Y. Yang and Z. Wang, "Functional characterization of an invertase inhibitor gene involved in sucrose metabolism in tomato fruit," *J. Zhejiang Univ. Sci.*, vol. 16, no. 10, pp. 845-856, 2015.