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Mutagenesis of Cellulose Synthase (*CesA*-Like) Gene in Tomato Using Clustered Regularly Interspaced Short Palindromic Repeat/CAS9-System

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Abstract

Background: During ripening, the changes in texture involved remodelling of cell walls of fruits including tomato and also alterations in tissue water relations caused by modifications in the cuticle. **Aims and Objectives:** To better understand the relationship between cell wall remodelling and fruit softening, an understanding of cell wall structure is necessary. **Materials and Methods:** Cellulose synthase gene that consists of cellulose synthase (*CesA*-like) plays important role in cellulose biosynthesis. However, *CesA* family genes are yet to be fully characterized in Solanaceae species. **Results:** In this study, we generated transgenic plants to test the role of *CesA* like gene in texture changes using tomato (Solanum lycopersicum) as a model system. We used the recently developed clustered regularly interspaced short palindromic repeat/Cas9 DNA editing technology to generate mutations in the target gene. **Conclusion:** Nevertheless, there was no mutation recovered in the *CesA*-like gene, and this indicates that this gene product is likely essential for regeneration of plantlets from tissue culture.

Keywords: Cell wall, cellulose synthase, clustered regularly interspaced short palindromic repeat-Cas09, gene editing, tomato

Introduction

The development of methods for genome editing has progressed rapidly in the last two decades. Several methods have been developed including the use of zinc finger nucleases[1] and transcription activator-like effector nucleases.[2] More recently, the clustered regularly interspaced short palindromic repeat (CRISPR) associated-Cas9 endonuclease has been used.[3]

According to Feng *et al*.,[4] CRISPR/Cas9 system successfully generated mutation using Cas9 driven from CaMV 35S promoter and the synthetic sgRNA from the AtU6-26 promoter in *Arabidopsis* or OsU6-2 promoter in rice. A customized sgRNA encoded by a sequence of ~100 nt is required to target a specific sequence. Cas9 does not have to be reengineered for each new target site. Thus, the sgRNA: Cas9 system is therefore much more straightforward than RNA interference. The first report for the use of CRISPR/Cas9 in tomato was by Ron *et al*.[5] and Brooks *et al*.[6] Ito *et al*.[7] also reported the efficiency of CRISPR/Cas9 method to silence the RIN gene where the mutations that contain insertion or deletion resulted in inhibition of fruit ripening.

Hence, the aims of this study are to investigate the function of the *CesA*-like gene in the transgenic tomato lines by generating a knockout using CRISPR/Cas9 DNA editing and examine the influence of this mutation on tomato fruit softening. In plants, most of cellulose synthase or *CesA*-like gene is involved during cellulose and hemicellulose biosynthesis. During fruit development, cellulose synthases are highly expressed in tomato and then decreased at the breaker stage.[8] They are likely to be involved in the biosynthesis of cellulose,[9] but till now not much studies regarding this gene is done in tomato (*Solanum lycopersicum*).[10]

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Materials and Methods

Plant materials

Tomato (*S. lycopersicum*) plants cv. Ailsa Craig, wild type and those containing CRISPR/Cas9 constructs were grown under standard glasshouse conditions with 16 h of day length and temperatures of 20°C during the daytime and 16°C at night. Supplemental lighting was supplied when required.

Vector construction for generating stable transgenic plants

Primers were designed [Table 1] using CesA-like sequence (Solyc08 g061100) from tomato genome browser at www.solgenomics.net. A specific sequence within the gene was then chosen to represent the guide RNA [Figure 1]. To generate the stable transgenic plants, constructs Level 1 (pICH47751::AtU6p::sgRNA, pICH47732::NOSp::NPTII-OCST, pIcH47742:35Sp::Cas9-NOST, Pich41766 Linker) were used and assembled to Level 2 (pAGM4723) through the Golden gate cloning method.[11]

Generation of stable transgenic plants

The final Level 2 Cas9/sgRNA construct was transformed into *Agrobacterium* EHA105 by electroporation. Method described by Smith *et al*.[12] was used to generate stable transgenic plant in growth room at 25°C with 16/8 h:light/dark photoperiod.

Identification of the transgenic lines

To identify the presence of T-DNA in the transgenic plant lines, a set of polymerase chain reaction (PCR) reactions was performed using *CesA*-like gene-specific primer and the NPTII::Cas9 primer. The DNA from wild-type Ailsa Craig was used as a positive control for the gene-specific primer pair and as negative control for T-DNA mutation. The sequence of transgene was confirmed by sequencing.

Identification of single copy homozygous lines

The T_0 lines were grown to fruiting and T_1 seeds were collected. Then, 10–12 T_1 seeds were then sown for each of the line. The DNA from each of the plants was extracted using DNeasy Plant Mini Kit (Qiagen). PCR was used to establish the presence of the Cas9 gene and also to obtain the edited *CesA*-like sequences. The PCR products were then sent for sequencing.

Results and Discussion

Vector construction of PSY1

According to the product sizes, most convenient primer products is 8CeSa061100 where the PCR amplicons of the target genes with the length as ~ 100 bp. This product was then cloned into vector pICSL01009::AtU6p (SpecR) and shown to be correct by sequencing. By using Golden Gate cloning approach, Level 1 constructs were assembled to Level 2 vector that involved pICH47751::AtU6p::sgRNA, pICH47732:NOSp: NPTII-OCST, pIcH47742::35Sp::Cas9-NOST, Pich41766 Linker and pAGM4723. The presence of DNA for CESA-like in Level 2 construct was confirmed using PCR. The destination vector Level 2 Cas9/sgRNA construct was then transformed into *Agrobacterium* EHA105 by electroporation. Figure 2 shows the PCR products that were amplified from the isolated plasmid DNA (Level 2) for samples *CesA-like* using NPTII/Cas9-specific primer.

Analysis of the CesA-like transgenic lines

There were seven transgenic plant lines regenerated using the *CesA-like* construct. These transgenic plants were then transferred to the glasshouse. To confirm the presence of the transgenes, DNA was extracted from leaves of the putative transformants. Primers NPTII/Cas9 was used to amplify the Cas9 region where the size of the product is 1600 bp [Figure 3]. The PCR generated an amplicon of the correct size of 1600 bp in putatively transformed plant lines No. 1, 2, 3, 4, 5, 6 and 7 for *CesA-like*. In addition, no PCR amplification product was recovered in WT plants. The experiment demonstrated that these lines contained the Cas9 gene, but it does not indicate if the endogenous gene has been mutated.

Validation of clustered regularly interspaced short palindromic repeat/Cas9 driven mutations in the CesA-like

A PCR coupled with restriction digestion strategy as well as sequencing was used to validate the presence of mutations in the target genes of T_0 lines [Figure 4a]. A PCR approach for the *CesA-like* gene was designed to give a single amplicon of 300 bp containing a *BssSI* site [Figure 4b]. The guide RNA would be expected to target sequences in the region of this restriction site. This would result modification of sequence at the site and prevent subsequent digestion by *BssSI*.

However, the *BssSI* digestion pattern of PCR-amplified DNA from the 7 individual T_0 plants shows similar patterns to wild

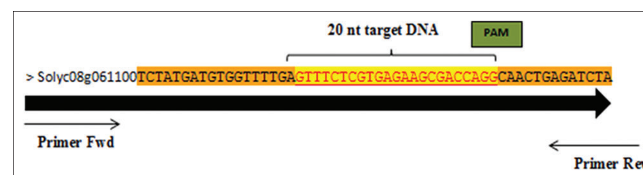

Figure 1: Cas9/sgRNA-mediated mutagenesis in *Solanum lycopersicum*. Scheme for Cas9/sgRNA-mediated mutagenesis of a nonfunctional (out-of-frame) mutant of Solyc08g061100 coding sequence

Table 1: Primer sequences of gene for cellulose synthase

Primer ID	Primer sequence 5' to 3'
8CeSa061100.F	TGTGGTCTCAATTGTCTTTTTGGGGCACAGCGGGGGTTTTAGAGCTAGAAATAGCAAG
9CeSa061100.F	TGTGGTCTCAATTGTTTCTCGTGAGAAGCGACCAGGTTTTAGAGCTAGAAATAGCAAG
10CeSa061100.F	TGTGGTCTCAATTGTTTCTGCAGTTCTTACCAATGGTTTTAGAGCTAGAAATAGCAAG
RevCRISPR	TGTGGTCTCAAGCGTAATGCCAACTTTGTAC

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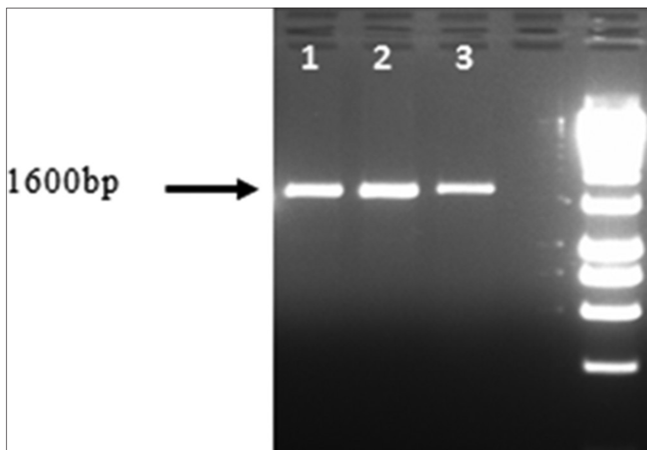

Figure 2: Polymerase chain reaction products were amplified using the Cas9/NPTII-specific primers from *Agrobacterium* EHA105. The purified polymerase chain reaction products were separated by electrophoresis on a 2% (w/v) agarose gel in 0.5X TAE at 100 V for 30 minutes. Lane 1, 2 and 3 are polymerase chain reaction products of sgRNA constructs which were isolated from colonies of *Agrobacterium* EHA105, Lane 4: Hyperladder marker

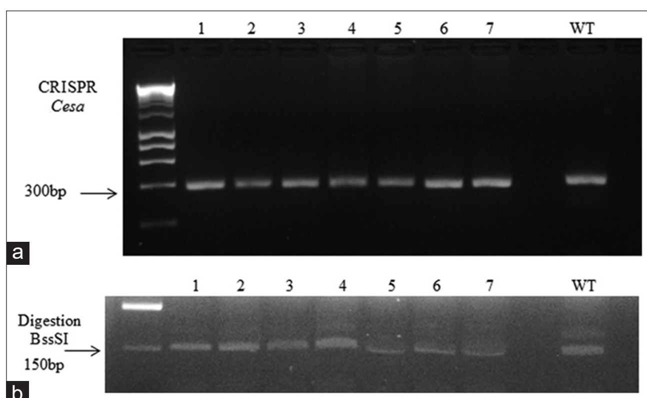

Figure 4: Efficiency of Cas9/sgRNA mutagenesis. (a) Polymerase chain reaction amplification of the clustered regularly interspaced short palindromic repeat: *CesA*-like lines to generate a 300 bp product that could then be challenged with *BssS1*. (b) Polymerase chain reaction/ restriction digestion analysis demonstrates that all products can be cut with *BssS1* indicating a lack of mutations in this sequence

type. All of the amplification products were digested by the enzyme. This suggests that the CRISPR/Cas9 targeting of the *CesA-like* gene has not been successful. Thus, to confirm this analysis, the PCR products from the seven T_0 were purified and sent for sequencing. The sequencing results confirmed the absence of mutations in the target site of *CesA-like* gene [Figure 5]. One explanation for these results is that the *CesA-like* gene is essential for proper regeneration of plants in tissue culture, and knocking out this gene is lethal.

Conclusion

The failure to regenerate CRISPR/Cas9-driven mutations in the *CesA*-like gene suggests that the gene is necessary for plant development. It also suggests that in some instances,

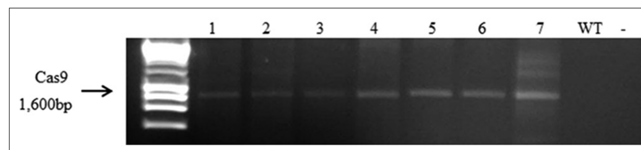

Figure 3: Polymerase chain reaction genotyping of seven representatives (a) CRISPR::CesA-like plants showing the transgenic lines that contain Cas9/ sgRNA gene construct in T_0 plants

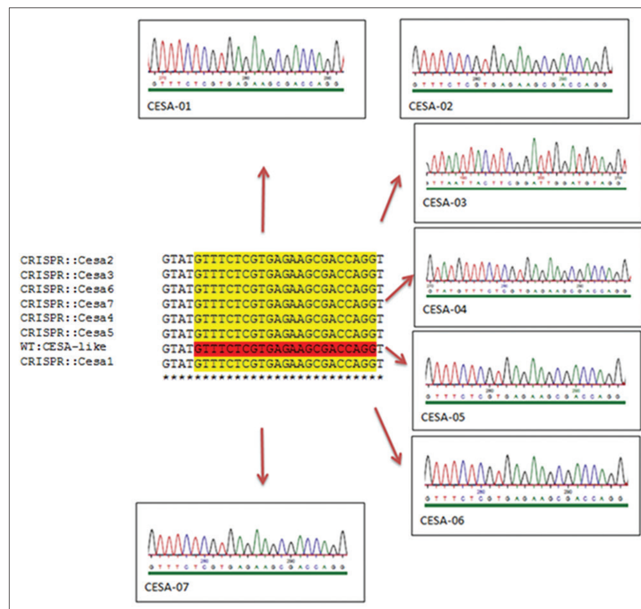

Figure 5: Confirmation of inheritance of a modified or nonmodified *CesA-like* gene. Sequence alignment and DNA sequencing traces from sequencing of polymerase chain reaction amplification of clustered regularly interspaced short palindromic repeat::*CesA*-like as target sites where there are no modification in the sequence level

CRISPR will not be the technology of choice for transgenic experiments where complete silencing of a target gene can be lethal.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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