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Cloning and CRISPR/Cas9-mediated targeted mutagenesis of *NtTRE* in *Nicotiana tabacum*

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Abstract: In order to study the function of trehalase in tobacco, the trehalase (EC 3.2.1.28) gene was cloned from Tobacco K326 (*Nicotiana tabacum*). CRISPR/Cas9 genome editing technology was applied to create the mutagenesis in NtTRE. Two targets were selected as the editing sites in the first extron of NtTRE. The pORE-CRISPR/Cas9 vector was constructed to edit target sequence. As a result, 15 transgene lines were obtained. Of them, there were 3 lines to be knocked out successfully. The first mutant is A inverted T on 285 site. The second mutant is A inverted T on 293 site, and the third were two mutation site, A inverted T on 285 and A inverted G on 295 site. All of these three mutations can cause NtTRE to be replaced by another protein. In this study, we produced mutants materials for further researching trehalose and NtTRE functions in tobacco.
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Keywords: trehalase; trehalose; CRISPR/Cas9; *Nicotiana tabacum*

1. Introduction

Trehalose (a-D-glucopyranosyl-1,1-a-D-gluco pyranoside) was first record in Selaginella lepidophylla [1]. It is present in green algae, mosses, liverworts and ferns, but its content is very low in angiosperms, such as flowering plants [2-3]. Trehalose is highly soluble and non-reducing disaccharide. It has the special physicochemical properties which effectively protect protein molecules from denaturalization and deactivation and maintain the organism vital processes and biological characteristics under some stress conditions, such as high temperature, cold, high osmotic pressure and dehydration [4-5]. Previous studies have proved that trehalose is associated with the disease resistance and stress response of plants [6-7], and it can enhance the tolerance of plants to biotic stress such as drought, low temperature, salt damage etc, and abiotic stress such as disease, insect pest and so on [8].

CRISPR/cas9 system (Clustered regularly interspaced short palindromic Repeats (CRISPR)/CRISPR associated 9) is a rapid genome editing technique that the Guide RNA binds to the target sequence of the gene [9]. Under the action of the cas9 endonuclease, the target DNA is cut and the DNA repair system is started, leading to the base to be replaced or deleted during the repair process. Replacement and deletion result in loss of gene function and error proteins.

In recent years, CRISPR/cas9 is widely used in the genome editing for creating mutagenesis to investigate gene function, such in rice, wheat and so on [10-12]. It was reported that trehalose could increase the resistance of TMV in tobacco. Trehalase (EC 3.2.1.28) hydrolyzes α,α′-trehalose into two glucose [13], which is a key enzyme for trehalose metabolism in plants [4,5,14]. The amount of trehalose might increase when the expression of trehalase is interrupted, which improve the tolerance of TMV in the tobacco. Therefore, the trehalase gene was cloned from tobacco G80 and mutated by CRISPR/cas9 technology. The trehalase gene knockout plants were obtained to study the mechanism of trehalose in the TMV resistance of tobacco.

2. Material and Methods

2.1 Plant material and treatment

Tobacco (G80) was planted in greenhouse. The leaves with good growth conditions were selected, washed with distilled water, dried, and wrapped with tin foil. The leaves were quickly frozen with liquid nitrogen and then stored at -80 ℃ for RNA extraction.

2.2 Total RNA extraction and reverse transcription

Tobacco leaves (about 100mg) stored at -80 ℃ were quickly frozen in liquid nitrogen. Total RNA was extracted according to the Trizol method. Then electrophoresis was used to analyze the quality of RNA. The cDNA was obtained according to the instructions of the reverse transcription kit.

2.3 Cloning of trehalase gene

The cDNA sequence of tobacco *trehalase* gene was searched on NCBI. The primers were designed based on the sequence, and the upstream and downstream primers were CDS-F and CDS-R. The primer sequences were as follows: CDS-F: ATGATTTTCACTCTGTTTATATT, CDS-R: TCAGTAGCAGTCAATCTTCA.

The cDNA obtained by reverse transcription was used as a template for cloning. The PCR cycle conditions were as follows: predenaturation at 94 ℃ for 5 min, denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s, extension at 72 ℃ for 92 s for a total of 30 cycles, 72 ℃ for a final extension of 10 min.

2.4 The *CRISPR/cas9* vector construction

According to the *trehalase* gene sequence, the primer design tool was used for selection of suitable target sites. The target site is mainly at the front of the gene coding region containing 20 bases, which is followed by the three bases NGG (N is an arbitrary base) as the PAM region. The target sites were named gDNA1 and gDNA2. The target sites primers were synthesized by shanghai shenggong Company and annealed into double DNA strand. The target double DNA strand was cleaved by Bsa1 and fused into *pORE-CRISPR/Cas9*. The target sites primers were:

gDNA1-F: GATTGCTTCAATCCTATGGCTACAA, gDNA1-R: AAACTTGTAGCCATAGGATTGAAGC, gDNA2-F: GATTGTTTATAGGCAAATATTTGAA, gDNA2-R: AAACTTCAAATATTTGCCTATAAAC.

The constructed vector was transformed into DH5α, and then the positive bacterial plaque was selected for sequencing and transformed into Agrobacterium GV3101 for infecting tobacco.

2.5 Tobacco transformation

The Agrobacterium containing *pORE-CRISPR/Cas9* vector infected tobacco leaf discs and then selected in MS solid containing NAA, 6-BA, Kana and Timentin. Finally, the positive plants were was obtained from the callus.

2.6 The checking of positive plants

The genomic DNA was extracted from the positive plants leaves and amplified by PCR using the identification primers, the primers were:

Kana-F: CAGGTTCTCCGGCCGCTTGG,

Kana-R: GGAGATCCTGCCCCGGCACT,

Cas9-2-F: CTCAACACAACATATACAAAACA, Cas9-2-F: CTTTGGCCATCTCGTTTGA.

The PCR production of *Cas9-2* was fused into T-vector and the single clone was selected to sequence for checking the mutation.

3. Results

3.1 Cloning of trehalase gene

Total RNA was extracted from tobacco leaves, and cDNA obtained by reverse transcription was used as a template. A 1743bp gene fragment was cloned by PCR (Fig.1), which was consistent with *trehalase* in tobacco, and the sequencing result was accorded with the bases sequence derived from the NCBI, indicating that *trehalase* gene has been successfully cloned from tobacco.

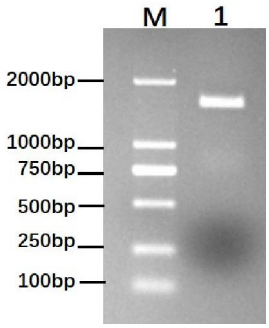

Figure 1. PCR results of *NtTRE*
M: DL2000, 1: *NtTRE*

3.2 The construction of *trehalase pORE-CRISPR/Cas9* vector

We selected two sites of the first extron in *NtTRE* as the gDNA to construct the mutation sites (Fig.2), the constructed *pORE-CRISPR/Cas9* vector was transformed DH5α, then the fragment about 500 bp was amplified by colony PCR, indicating that the colonies may be positive clones. The bacterial clones were selected for sequencing, and the sequencing results (Fig.3B) showed that 20 bp nucleotides was inserted into the *CRISPR/cas9* vector, indicating that the target site was successfully inserted into the *pORE-CRISPR/Cas9* vector and the knockout vector was successfully constructed (Fig.3).

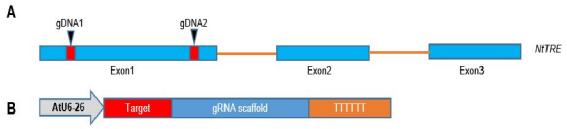

Figure 2 The target gDNA sites of *NtTRE* and the strategy for constructing *pORE-CRISPR/Cas9* vector

The target sites were CTTCAATCCTATGGCTACAA (gDNA1) and TTTATAGGCAAATATTTGAA (gDNA2).

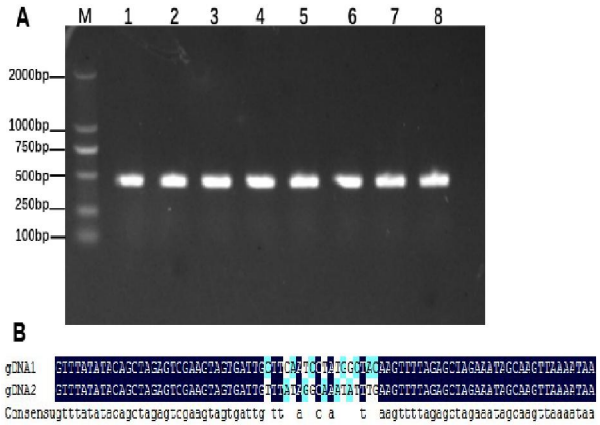

Figure 3 the colony PCR and sequencing were used to check the constructing of *pORE-CRISPR/Cas9* vector

3.3 Detection of the knockout transgenic tobacco plants

The T0 positive transgenic tobacco plants, obtained by Agrobacterium-mediated tobacco disc transformation, were detected by PCR. The genome DNA was extracted from the leaves of positive lines, PCR result showed that 100bp of the *kana* gene and 600bp of *Cas9* fragments were amplified, indicating the knockout *pORE-CRISPR/Cas9* vector was successfully transferred into the G80 plants.

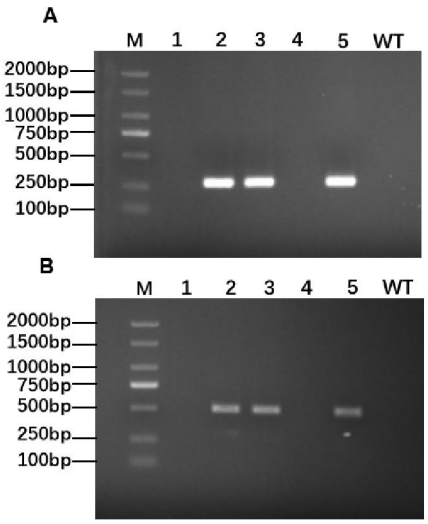

Figure 4 The vector fragment of *kana* gene (A) and *Cas9* (B) were used to check the transgene plants

3.4 Identification of knockout transgenic plants

In the T0 generation, the most positive transgenic plants were green, which is as normal as other tissue culture seedlings. Using the genomic DNA of knockout transgenic lines as template, *trehalase* gene were amplified by PCR. 15 plants were detected, and the fragments of *trehalase* were amplificated successfully in the positive lines. In the 15 plants, only 3 were identified as the mutations. One is A inverted T on 285 site in the target site, the other is A inverted T on 293 site in the target site, and the third were two mutation site, A inverted T on 285 and A inverted G on 295 site near the target site. All of these three mutations can cause the right protein replaced by another protein (Fig.5). Based on the result, we obtained three *trehalase* mutated lines.

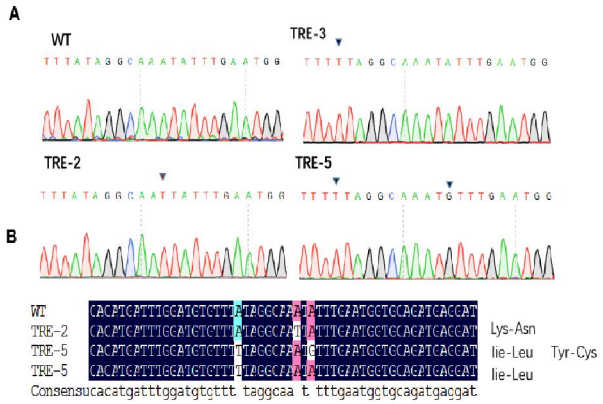

Figure 5 the mutation sites of the positive transgene plants

4. Discussion

Trehalose is important during plant growth and development, which is involved biological stress responses [4,6]. This stable sugar in cells can stabilize membranes under abiotic stress [6-7]. Despite plants benefits from trehalose in the biological procession, there is little known about its metabolites function. Tobacco is an important economic crop and bring huge economic benefits through improving tobacco resistance. Trehalase is a key enzyme for trehalose metabolism in plants [4]. The knocking out of *trehalase* gene may increase the content of trehalose, improving the resistance of TMV infection.

Therefore, the *trehalase* was cloned from G80 based on the sequence GenBank (GenBank: AB501123.1). The coding region of the *trehalase* is 1743 bp, which encodes 580 amino acids. The CRISPR/cas9 technology was applicated to knockout *trehalase* in tobacco. After the deletion of the *trehalase* gene, 3 independent knockout lines were obtained. These mutants provide valuable materials for the study of trehalose metabolism and revealing the role of trehalose in plant stress resistance.

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