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Efficient Transposition of the Retrotransposon *Tnt1* in Cucumber (*Cucumis sativus* L.)

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A B S T R A C T

Tnt1 is an active retrotransposon originally identified in tobacco (*Nicotiana tabacum* L.) (Grandbastien et al., 1989), but its transposition activity could be activated through tissue culture in other plant species. The insertions are stable and inheritable in the progeny, which has made it a valuable and versatile tool for developing insertional mutagenesis libraries in several plant species. Here, we explored its utility for mutagenesis in cucumber (*Cucumis sativus* L.). T_3 *Tnt1* transgenic cucumber plants were subjected to tissue culture to regenerate self-pollinated progeny. With PCR and analyses and Southern hybridization, we found regenerated plants maintained the original *Tnt1* insertion and created new insertions suggesting characteristic re-transposition activity of *Tnt1* during this process. Using genome walking, some flanking sequences of *Tnt1* insertions were recovered in regenerated plants. The results demonstrated that *Tnt1* could be stably inherited and re-transposable during tissue culture in cucumber and that it is feasible to use for developing an insertional mutagenesis library for cucumber.

Keywords: cucumber; *Tnt1*; retrotransposon; tissue culture; mutagenesis library

1. Introduction

Tnt1 is an active retrotransposon from tobacco (*Nicotiana tabacum* L.), which can randomly insert itself into the genomes of plants through its retrotransposon activity by tissue culture. Therefore, *Tnt1* can be used in functional genomic research of plants as a gene labeling technology (Tang and Ma, 2005). With improvements in *Agrobacterium*-mediated transformation and *Tnt1* insertion mutagenesis, *Tnt1* has been successfully used for mutant library construction in several plant species such as *Arabidopsis thaliana* L., *Lactuca sativa* Linn., *Medicago sativa* Linn., potato (*Solanum tuberosum* L.) and soybean (*Glycine max* L.) (Lucas et al., 1995; Courtial et al., 2001; Mazier et al., 2007; Tadege et al., 2008; Cui et al., 2013; Saowapa et al., 2013).

Cucumber (*Cucumis sativus* L.) is one of the most important vegetables around the world. Since the public release of its draft genome (Huang et al., 2009), research has gradually come to shift from structural to functional genomics. However, like many other horticultural crops, the number of cucumber mutants available is limited. This hinders understanding of functions of horticulturally important genes. Several EMS-based mutagenesis studies have been published for cucumber (Hu et al., 2011; Wang et al., 2014), the efficiency of EMS mutagenesis seems low. Thus, in this study, we tested the effectiveness of the tobacco-based *Tnt1* retrotransposon mutagenesis system in cucumber. Self-pollinated progeny (T_3) of *Tnt1* transgenic cucumber plant of the inbred line '9930' were subjected to tissue culture to obtain regenerated plants. Genome walking was utilized to analyze the flanking

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sequences of *Tnt1* insertions in regenerated plants. Southern hybridization was used to reveal and validate *Tnt1* re-transposition activities in the cucumber genome. We found that *Tnt1*-based retrotransposon system to be a useful tool in mutagenesis library construction in cucumber for functional genomics studies.

2. Materials and methods

2.1. Plant material

Two T_2 transgenic cucumber lines, NCT05 and NCT10, containing the retrotransposon *Tnt1* were used as materials in Cucumber Laboratory, Shanghai Jiao Tong University, in December, 2016. These were originally developed by the Cucumber Improvement Program at USDA-ARS, Horticulture Department of the University of Wisconsin. The *Tnt1* contains the long-terminal repeat (LTR) sequence on both sides (5′UTL and 3′UTL of *Tnt1*). NCT10 and NCT05 were originally derived from the transgenic plant of '9930' (T_0) by Tnk23 vector (d'Erfurth et al., 2003). There were at least 4 copies of *Tnt1* in the T_0 plant. Specific primers, Itr1/Itr2 from the LTR, and Km3/Km5 within the *NPTII* (kanamycin resistance) gene of the T-DNA vector (d'Erfurth et al., 2003) were used in PCR to analyze T_1 plants. Plants that carried *Tnt1* but not *NPT II* were self-pollinated to produce T_2 lines, NCT10 and NCT05. For subsequent experiments, plants of the two lines were verified for the presence of *Tnt1* with PCR with Itr1/Itr2 primers, and selfed. Seeds of the two T_3 progeny, NCT10-1 to -10 (NCT10-1–10) and NCT05-1 to -10 (NCT05-1–10), were employed for tissue culture in this study.

2.2. PCR detection for Tnt1 insertion

Genomic DNA from leaves of transgenic and control (regular '9930') plants were extracted by CTAB and used as the templates for PCR employing *Tnt1* specific primers (Itr1/Itr2). Each PCR was conducted in a 20 μL reaction containing 50–100 ng DNA, 1 μL each of forward and reverse primer, 10 μL 2 × PCR Solution Premix *Taq*™ (TaKaRa, China) and 6 μL ddH_2O. The PCR was carried out using the following program: pre-denaturation at 95 °C for 10 min, then 28 cycles for heat denaturation (95 °C, 30 s), annealing (57 °C, 30 s) and extension (72 °C, 2 min). PCR products were separated with 2% agarose gel electrophoresis.

2.3. Tissue culture of transgenic plants

T_3 seeds from two T_2 plants, NCT10-1–10 and NCT05-1–10, were used for tissue culture to examine transposition activity of *Tnt1*. The regenerated plants were labeled as T10-1–10-x, respectively. Seeds were soaked in 65 °C water for 3 h. The seed coats were then removed with tweezers. The seeds were surface disinfected by washing in 75% ethanol for 30 s, 1% sodium hypochlorite solution for 10–15 min and rinsing for 3 times in sterilized distilled water (Wang et al., 2006; Ren et al., 2014). Sterilized seeds were placed on the MS medium and kept at (25 ± 2) °C for 48 h in dark until the inner integument of seeds fell off (Fig. 1, A). Then, the triangle segment where the hypocotyl met the cotyledons was connected. It was approximately 1/3 to 1/2 distal of the cotyledon were carefully removed with a sterilized scalpel and a tweezer (Fig. 1, B); the remaining cotyledon segments were transferred to the induction medium (MS + 0.5 mg·L^{-1} 6-benzylaminopurine + 1.0 mg·L^{-1} abscisic acid + 30 g·L^{-1} sucrose + 2.4 g·L^{-1} phytagel, pH 5.8) for culture at (25 ± 2) °C, 16/8 h (light/dark) photoperiod. After 10–15 days, the segments with regenerated buds were transferred to MS medium (MS + 30 g·L^{-1} sucrose + 2.4 g·L^{-1} phytagel, pH 5.8) for shoot elongation (Fig. 1, C and D). The shoots, which were ≈2 cm long, were cut off for rooting in MS medium (Fig. 1, E and F). Finally, regenerated plants were acclimatized and grown in sterilized matrix in a greenhouse (Fig. 1, G and H).

Table 1 Primers used in this study

Primer	Sequence (5′→3′)	Location
PP1	GGTGTTGTCCCATCCAGAAAAG	Open reading frame of *Tnt1*
PP2	CCTTGGTGCCTGCTTCAATC	Open reading frame of *Tnt1*
SP1	GCGGAATAATAATGTAGCACCGAGATAC	5′LTR of *Tnt1*
SP2	GACCCCGAGAGGAGCAACTGAATC	5′LTR of *Tnt1*
SP3	CCCACTCACTACAATATCGCTCACTC	5′LTR of *Tnt1*
In2	CGAAGAACATCAAGAAGCACC	Chr2 of cucumber genome
In3	CAATGAAGGTGCAGTGTCA	Chr3 of cucumber genome
In4	CTCACTCATCCAATTCCCTCATG	Chr4 of cucumber genome
In5	GGGTTAGTAGATAGGTTGTTCTC	Chr5 of cucumber genome
Itr3	AAATGTGACAAAAAATTCGTACCT	5′LTR of *Tnt1*
Itr4	GAGAGGAGCAACTGATATCACT	5′LTR of *Tnt1*
Itr5	TTCCCACCTCACTACAATATCG	5′LTR of *Tnt1*

2.4. Southern hybridization

Southern hybridization was used to verify *Tnt1* transposition and estimate *Tnt1* copy numbers in the genome in NCT10 and NCT05 plants and their T_3 progeny. A fragment within the *Tnt1* open reading frame was PCR amplified using the primer pair PP1/PP2 (Fig. 2; Table 1) and used as the probe in Southern hybridization. The PCR products were recovered and DIG-labeled using a DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche, Switzerland). Labeled probes were purified by passing through G-50 columns following manufacturer's instructions.

For Southern hybridization, genomic DNA of randomly selected NCT10 and NCT05 plants (T_2), T10-4−x, T10-5−x, and non-transgenic control '9930' plants were isolated with CTAB. Each DNA sample, all of which were ≈2 μg in volume, was digested with *Eco*R I at 37 °C for 10 h. The digested product was subjected to 0.8% agarose gel electrophoresis overnight at 25 V. The gel was rinsed with 0.2 mol·L^{-1} HCl for 10 min for depurination and denatured twice for 15 min each time with denaturation solution (20 g·L^{-1} NaOH + 87.6 g·L^{-1} NaCl). DNA was transferred to nylon membrane pre-treated with the alkali transfer solution (16 g·L^{-1} NaOH + 58.44 g·L^{-1} NaCl) for 5 min. The nylon membrane with DNA samples was rinsed with hybridization solution, placed in a hybridization tube, and hybridized with the denatured probe according to the instructions provided by the manufacturer. Hybridization images were taken by exposing the membrane to an X-ray film.

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