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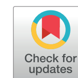
Expression of T7-based constructs in tobacco cells



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Bacteriophage T7 promoter and RNA polymerase (T7-Pol) are widely used for recombinant protein expression in bacteria. In plants, there exists conflicting results regarding the efficacy of protein expression from T7-Pol-derived mRNAs. To reconcile these contradictory observations, the expression of green fluorescent protein (GFP) from T7 constructs was evaluated in tobacco protoplasts. T7 constructs transcribed by a nuclearly targeted T7-Pol did not express GFP in plant protoplasts, however T7-Pol lacking a nuclear targeting signal was able to translate cytosolically transcribed mRNAs, but only if the messages contained a viral translation enhancer. GFP expression was further evaluated at the plant level by using agroinfiltration-mediated transient expression system. Unlike for cytosolic expression, nuclear T7 transcripts containing a viral translation enhancer element did not express GFP, and modifications designed to stabilize and facilitate export of T7 transcripts to the cytosol did not improve the expression. We conclude that expression of nuclear T7 constructs is not feasible in tobacco cells, but cytosolic transcription provides an alternative means to over-express RNAs directly in the cytosol.

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1. Introduction

Bacteriophage T7 promoter and T7 RNA polymerase (T7-Pol) have been used widely to express recombinant proteins in bacteria and are one of the most important systems for protein production [1]. This T7 promoter/Pol combination has also been assessed for recombinant protein expression in yeast and mammalian cells. In both cases, a reporter gene construct under the control of a T7 promoter was efficiently transcribed by T7-Pol that was targeted to the nucleus. However, efficient reporter protein expression was observed in mouse cells, but not yeast cells [2,3]. The latter null result was further supported by other studies of mammalian cells which determined that nuclear T7 transcripts are uncapped and retained in the nucleus, thereby preventing translation [4–6]. To overcome the nuclear retention barrier, cytosolically localized T7-Pol was used to transcribe reporter genes from DNA plasmids residing in the cytosol [4,5]. Additionally, T7 transcripts were designed to include a viral internal ribosomal entry site (IRES) from encephalomyocarditis virus to facilitate cap-independent translation [4,5]. These modifications significantly improved reporter protein expression in these [4,5] and other T7 promoter/Pol-based mammalian systems [7,8].

Two laboratories have previously investigated reporter protein expression from mRNA transcribed by T7-Pol in the nucleus of tobacco cells, with contrasting results. The earlier study showed that reporter protein from nuclearly targeted T7 constructs were inefficiently expressed in tobacco plants [9], while the latter study found efficient expression of nuclearly localized T7 constructs in tobacco plants [10]. Based on these conflicting results, the utility of T7-based expression of heterologous proteins in plants remains uncertain.

In this report, the expression of T7-based constructs was initially evaluated in plant cells, and the results revealed that nuclearly transcribed T7 constructs do not express GFP. In contrast, cytosolically transcribed T7-Pol mRNAs that also contained a plant virus translational enhancer (TE) efficiently mediated GFP expression. Expression from T7 constructs containing a TE was also investigated at the whole plant level, via transient agroinfiltration. No reporter protein expression was observed from TE-containing mRNAs, further supporting the concept that expression of nuclear T7 constructs is not feasible in plant systems.

2. Materials and methods

2.1. Plasmid construction

Plasmids containing poly-adenosine tracts were maintained in DH5α strains below 30 °C to avoid truncation of A-tracts.

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pT7-GFP: PCR product 1 was generated by using a primer set (pT7-11-1 and pT711-2) without a template, and PCR product 2 was made by using pESC-Leu-GFP-VHL [11] and a primer set (pCaMV4 and pT7-12). PCR product 1 (digested with SbfI and NcoI) and PCR product 2 (digested with NcoI and SbfI) were triple-ligated into pRTL2 [12] cut with SbfI.

pT7-GFP-Tφ: PCR product 1 was generated by amplifying T7 termination signal sequence with SacI and HindIII sites at each end, and PCR product 2 was generated by amplifying T7 termination signal sequence with Hind III and EcoRI sites at each end. These two PCR products, digested with SacI/HindIII and HindIII/EcoRI, respectively, were triple-ligated to pHST20 TBSV [13] cut with SacI and EcoRI, generating pHST20 TBSV Tt. Using this plasmid as a template, PCR product 3 was made using the primers pT7-14 and pT7-15.

PCR product 4 containing GFP ORF was amplified using pT7 GFP and a primer set (pT7-11-1 and pT7-13). The PCR product 3 above (digested with SacI and SbfI) and PCR product 4 (digested with SbfI and SacI) were triple-ligated to pRTL2 cut with SbfI.

pCaMV-GFP: 35S CaMV promoter sequence is amplified using pRTL2 as a template and a primer set (pCaMV1 and pCaMV2), and digested with SbfI and NcoI. Also, GFP ORF was cut out from pT7 GFP using NcoI and SbfI. 35S CaMV promoter and GFP ORF were triple ligated to pRTL2 cut with SbfI. For the generation of p2xCaMV GFP, dual 35S CaMV promoter was amplified by PCR and was similarly triple ligated to pRTL2 cut with SbfI.

pRTL2-nT7-Pol or pRTL2-cT7-Pol: T7-Pol cDNA with or without nuclear localization signal was amplified using a standard PCR, and inserted to pRTL2 cut with SalI and NheI.

pTBSV-sg2-GFP: For pTBSV-sg2-GFP, T7 promoter, sg2 5′ UTR (nucleotides, 3842–3889), GFP ORF, and the 3′ UTR of TBSV genome were fused. The overlapping PCR products were ligated to pHST20 TBSV Tt cut with SacII and SmaI.

pRTL2-GFP: GFP ORF was amplified using pESC-Leu-GFP-VHL and a primer sets (pTrans12-1 and pTrans13-1), digested with XbaI and NheI, and inserted to pRTL2 (cut with the same enzymes).

pTEV-GFP: T7 promoter and TEV 5′ UTR were amplified using pRTL2 GFP and a primer set (pTEV1 and pTEV2), and digested with SbfI and NcoI. GFP ORF and CaMV poly A signal sequence were cut out from pRTL2 GFP using NcoI and SbfI. The PCR product, GFP ORF and CaMV poly A signal sequence were triple ligated to pRTL2 cut with SbfI, creating pT7 TEV GFP CaMV polyA sig. Then T7 promoter, TEV 5′ UTR and GFP ORF were amplified using this plasmid and a primer set (pTEV3 and pTEV 4-1). After digesting the PCR product with SacII and SspI, it was inserted to pHST20 TBSV Tt cut with SacII and SmaI.

T7-TEV-GFP-A50: T7 promoter, TEV 5′ UTR and GFP ORF were digested out from pT7 TEV GFP CaMV poly A sig using SbfI and NheI. Poly adenosine residues, the ribozyme sequence, T7 termination signal were digested out from pTEV GFP using NheI and PvuII. These two digested DNAs were triple ligated to pCambia 0305 cut with SbfI and AfeI.

T7-CTE-TEV-GFP-A50: CTE sequence [14] was amplified and inserted to T7 TEV GFP A50 cut with ApaI.

2.2. Protoplast preparation and transfection

Tobacco protoplasts were isolated from tobacco plants grown for 2–3 months in green house at ambient room temperature (RT), and they were transfected with the plasmids according to the previously described method [15] with slight modifications. 1×10^5 protoplasts in 200 μl of MMG buffer were transfected with a total of 40 μg of plasmid(s) and 225 μl of 40% PEG solution. For the transfections with two plasmids (e.g., pT7 GFP and pRTL2 nT7 pol), 20 μg of each plasmid were used. The transfected protoplasts were incubated at 25 °C for ~24 h in the dark before the fluorescence microscopy analyses (Zeiss Axiocam IC fluorescence microscope, Zen software).

2.3. Agroinfiltration

Tobacco plants were agroinfiltrated as previously described [16] except that OD_{600} of agrobacterium was adjusted to 0.5. The agrobacterium used was AGL1. After agroinfiltration, the plants were grown for four days in a growth chamber (20 °C for 8 h in the dark and 23 °C for 16 h with light), before the fluorescence microscopy analyses.

2.4. Isolation of RNA and extraction of proteins

The agroinfiltrated areas of tobacco leaves were cut out and ground using liquid nitrogen, and the ground power was stored immediately at −80 °C until use. For isolation of RNA, about 1/5 full of the ground power in eppendorf tubes was mixed with 1 ml of Trizol solution and left for 15 min at RT with occasional vortexing. 200 μl of chloroform was then added and left for additional 10 min. The samples were centrifuged at 12,000 g for 20 min at 4 °C, and the upper phase was precipitated with 1 ml of isopropanol overnight at −20 °C. After centrifugation at 16,000 g for 20 min, the pellet was dried, dissolved in 100 μl water, and extracted with PCI (phenol: chloroform: isoamyl alcohol) three times. The upper phase was then ethanol-precipitated, washed and dissolved in 50 μl of water. The isolated total nucleic acids containing 1× T7 transcription buffer (Epicentre) were digested with 2 μl of DNase I (Epicentre) for 20 min at 37 °C, and the samples were PCI-extracted three times and ethanol-precipitated overnight. After centrifugation and washing the pellet with 70% ethanol, the pellet was dried and resuspended in 20 μl of water.

For extraction of proteins, equal volumes of the ground power and 2× SDS sample buffer were mixed and heated to 100 °C for 3 min before loading to SDS-PAGE. Western blotting was carried out using rabbit anti-GFP antibody (Cell Signalling) and secondary antibody conjugated with Cy3 (Jackson ImmunoResearch).

2.5. RT-PCR

Reverse transcription was carried out following the manufacturer's protocol (Superscript IV, ThermoFisher Scientific). PCR reactions were performed using fusion polymerase and its protocol (New England Biolabs). For the detection of GFP transcripts, PCR was done using the primers, pTrans 12-1 and pTrans 13-1. For the detection of T7 transcripts, the primers pT7 downstr and pTrans13-1 were used. To detect actin mRNA, the primers pToAct1 and pToAct2 were used.

2.6. The primers used in this study

pT7-11-1:
gagaaacctgcaggtaatacgactcactataggacacgctgaagctagtcgactc
pT7-11-2:
cttcagaaatcaacttttgttccatggctcgaggctagagtcgactagcttcagcg
pT7-12: cttgcatgcctgcaggtcactggattttg
pT7-13: gttcttgagctcaatagagagagatagatttgtagag
pT7-15: gttcttcctgcaggcagctatgacatgattacgaattc
pCaMV1: gaacaacctgcaggtgagacttttcaacaaagggtaatatc
pCaMV2: gttcttccatggctcgaggctagagtcgacthagcttcagcgtgtcccctct ccaaatgaaatgaac
pCaMV4: gatcaaccatggtgagcaagggcgaggagctg
pTrans 12-1: gatcaatctagaatggtgagcaagggcgaggagctgttc
pTrans 13-1: ttgatcgctagcttacttgtacagctcgtccatgc

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