Expression of Human Soluble Trail Protein in Transgenic Tobacco Nc89

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Abstract

Plant bioreactors have been considered as an efficient system for biopharmaceutical production. It offers many advantages, such as low cost of production, eukaryotic expression way and absence of human pathogens. In this study, we tried to express the soluble extracellular domain of human tumor necrosis factor related apoptosis-inducing ligand (sTRAIL) in transgenic plants of NC89, a tobacco variety that was planted widely. Using the chloroplast-targeted expression vector pGTPsT, which was constructed previously, we transformed NC89 tobacco leaf explants with the mediation of \textit{Agrobacterium tumefaciens} LBA4404. We used kanamycin resistance to select transformants and PCR analysis to confirm the presence of sTRAIL gene. Then, we performed RT-PCR analysis to detect the transcription of sTRAIL gene and Western blot to analyze sTRAIL protein accumulation in the leaves of the transformed tobacco plants. The results showed that sTRAIL was successfully inserted into NC89 genome of 8 independent transgenic lines and was expressed in all tested lines. However, no significant protein accumulation was detected at present analysis conditions. This suggests that the accumulation of sTRAIL in transgenic plants of NC89 is much lower than that in transgenic Petit Havana, another variety, which we have investigated previously. NC89, therefore, may not be a suitable plant variety used as plant bioreactor for foreign protein expression and accumulation. A strong protein degradation system may exist in the chloroplast of this variety. However, this point should by experimently tested in the future study.

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Keywords: chloroplast targeting expression; tobacco variety; NC89; sTRAIL; transgenic tobacco

1. Introduction

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Plants have been regarded as bioreactors for foreign proteins production, especially for pharmaceutical proteins. Plant systems are more economical in that pharmaceutical proteins can be produced in a larger scale than the expensive industrial methods by fermentation of bacteria, yeast or cultured animal or human cell lines. Another important advantage of plant systems is lack of contaminants from potential human pathogens as opposed to bacterial and mammalian expression systems, therefore it is safe for pharmaceutical production [1]. In addition, plants have the ability to carry out post-translational modification to proteins from eukaryotes.

TRAIL (tumor necrosis factor (TNF)-related apoptosis-inducing ligand) was firstly identified by Wiley in 1995 [2] and has been considered as a promising medicine in biologically-targeted anti-cancer therapy. The clinical trials for this protein is currently in phase II and may play an important role in future cancer therapy [3]. However, since the source of TRAIL is limited, recombinant biotechnology is needed to meet the demands of its production and research. Recombinant research results show that the carboxylic terminal 114-281 amino acids of TRAIL protein (sTRAIL) is soluble and as effective as the whole TRAIL protein [4], and therefore, how to elevate the production of sTRAIL has become the main question to be solved.

Expressing sTRAIL protein in tobacco chloroplasts is a significant attempt in sTRAIL production. Chloroplast is a semi-independent organelle in plant cell, in which foreign proteins can be protected from degradation of protease in cytosol. The protein expression level can also be elevated via polyploidy of chloroplasts. In this study, sTRAIL protein was targeted into plant chloroplast by transit peptide of ribulose-1, 5-bisphosphate carboxylase (Rubisco) small subunit from Pisum sativum, which was used successfully in other reports before [5,6]. Tobacco variety NC89 was chosen for transgene experiment. NC89 is a widely planted tobacco variety and the production is stable every year via routine planting technology [7]. In addition, NC89 has been used as a good transgene receptor in other reports [8]. sTRAIL expressed in tobacco NC89 may be easier to be spreaded into filed planting.

2. Materials and Methods

2.1 Experimental materials

Tobacco variety, NC89 was grown in sterile conditions on MS medium at 25°C under a 16 hours photoperiod on MS medium containing 3% (w/v) sucrose and 0.8% (w/v) agar and subcultured every 3-4 weeks. Plasmid pTrail containing strail gene was kindly provided by Prof. Dexian Zheng (Chinese Academy of Medical Sciences & Peking Union Medical College, China). pGN expression vector, containing cauliflower mosaic virus (CaMV) 35S promoter and selective marker neomycin phosphotransferase II gene(nptII) expression cassette, was kept in our laboratory. E. coli DH5α and Agrobacterium tumefaciens LBA4404 were hold in our lab.

2.2 Expression vector construction

Expression vector was constructed as the process mentioned in another report [9].

2.3 Antibiotic selection analysis

Leaves of NC89 sterile seedlings were cut into squares on RMOP medium containing 0mg/L, 50mg/L, 100mg/L, 150mg/L Kanamycin for antibiotic selection analysis.
2.4 Agrobacterium mediated transformation

Young leaves of NC89 were used for transformation. Expression vector pGTPsT was transformed into Agrobacterium tumefaciens LBA4404 by Freeze-thaw method. Tobacco leaves were cut into squares about 5mm × 5mm, and infected with Agrobacterium, for 15 min. Explants were co-cultured at 25°C in dark, and then transformed to selection medium, RMOP, containing 150mg/L kanamycin and 300mg/L cefotaxime. Regenerated shoots were rooted on MS medium containing 250mg/L kanamycin and 300mg/L cefotaxime.

2.5 PCR analysis

Total DNA from transformed and untransformed lines o was extracted by CTAB method. Primers specific to strail gene were used to detect the presence of foreign gene.

2.6 RT-PCR analysis

Total RNA was extracted with TRNzol extraction buffer (TIANGEN Biotech., Beijing, China) following the protocol. RNA was used as template in PCR to avoid contamination by DNA. RNA was reversely transcribed to cDNA by M-MLV (promega). Primers used in RT-PCR analysis were the same as that in DNA insertion test.

2.7 Western blot analysis

Total soluble protein (TSP) was extracted from 100 mg leaves of wild type and transgenic tobacco as the method in Oye M. 40 μg TSP per sample was separated on 15% SDS-containing polyacrylamide gels for 30 min at 100 V, and then 3 h at 150 V, then transferred to a methanol-treated PVDF membrane (Millipore) by electro blotting at 100 mA for 1 h. The membrane was blocked with 5% (w/v) milk in TBST buffer for 1 h at room temperature. Then the membrane was incubated in TBST solution containing a 1:1000 dilution of commercially available rabbit anti-TRAIL polyclonal antibodies (Sigma) for 2h at room temperature. Wash the membrane for 15min, 4 times with TBST buffer at room temperature. Incubate the membrane in 1:5000 TBST dilutions of alkaline phosphatase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) for 1 h. Then wash the membrane again as the method above. The incubation with Pro-light HRP lighting substrate (TIANGEN Biotech., Beijing, China) was performed and fluorescence signal was explored to X-ray films (FUJIFILM).

3 Results

3.1 Kanamycin selection analysis
Before transgene analysis, it should confirm which antibiotic concentration can inhibit plant growth. Four concentration grads were designed for NC89 explants. Results showed that 150 mg/L kanamycin can inhibit the regeneration of explants (Fig.1). So 150 mg/L of kanamycin was used for selection of shoots regenerated from transgenic tissues.

3.2 Vector construction and regeneration of transgenic lines

As showed in Fig.2, transformation vector was constructed as described previously [9]. pGTPsT vector contains npt II gene which confers transgenic tobacco kanamycin resistant. rbcS transit peptide encoding sequence was linked to the upstream sequence of strail fragment.

After transformation, regenerated resistant shoots were selected on medium containing 150 mg/L kanamycin (Fig. 3). 20 independent lines were obtained.
3.3 The presence of strail gene

PCR analysis was performed to detect the presence of *sTRAIL* gene in transgenic lines. 8 independent lines were confirmed to have the insertion of foreign DNA (Fig. 4).

3.4 Transcription of strail gene

3 transgenic lines were selected for transcriptional detection. All RNA samples extracted from transgenic lines were used as templates of PCR to detect if there are residues of genomic DNA, and no specific bands could be amplified (data not show). Then RT-PCR analysis was performed to test the transcription of *sTRAIL* gene. The results showed that *sTRAIL* was transcribed in all detected lines (Fig. 5).
Lane C+: Positive control using plasmid as template; Lane WT: Wild type tobacco; Lane N1,N3,N7: Transgenic lines; Lane M: Marker DL2k.

3.5 Protein accumulation analysis

The expression of sTRAIL protein was analyzed by Western blot analysis, but no sTRAIL protein was detected.

3 Discussion

Pharmaceutical protein production in plants has lower cost and is more safety than other bioreactors as a whole. Plants have a large amount of biomass. Take tobacco for example, up to 40 metric tons of leaves can be harvested from 1 acre [10]. In our previous study, we have obtained transgenic tobacco plants from the Petit Havana variety using the similar protocol and the sTRAIL protein level was estimated to approach 0.3% of the total soluble protein in leaves [9]. Therefore, the production of sTRAIL protein from tobacco leaves of 1 acre could achieve kilograms, which would have a commercial value over 1 billion dollars.

However, the accumulation of foreign proteins in plant cells has to be improved. In this study, we have tried to express sTRAIL in another tobacco variety NC89. Our results showed that the encoding sequence for sTRAIL was successfully inserted into nuclear genome (Fig. 4) and that its transcripts was also detected (Fig. 5). Unfortunately, different from transgenic plants of the Petit Havana variety, we have not detected sTRAIL protein by Western blot. Although NC89 is a widely planted tobacco variety, it may not be suitable as a plant bioreactor to express pharmaceutical proteins. The chloroplasts of NC89 plant might have degradating enzymes that could protect the plants from accumulation of foreign proteins. Nevertheless, this suggestion should be confirmed experimentally in the future study. All authors must sign the Transfer of Copyright agreement before the article can be published. This transfer agreement enables Elsevier to protect the copyrighted material for the authors, but does not relinquish the authors' proprietary rights. The copyright transfer covers the exclusive rights to reproduce and distribute the article, including reprints, photographic reproductions, microfilm or any other reproductions of similar nature and translations. Authors are responsible for obtaining from the copyright holder permission to reproduce any figures for which copyright exists.

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References

[1] Daniell H. Production of biopharmaceuticals and vaccines in plants via the chloroplast genome. *Biotechnol J* 2006; 1:1071-9.

[2] Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, et al. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* 1995; 3: 673-82.

[3] Holoch PA, Griffith TS. TNF-related apoptosis-inducing ligand (TRAIL): a new path to anti-cancer therapies. *Eur J Pharmacol*, 2009; 625: 63-72, December 2009.
[4] Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 1999; 104: 155-62.

[5] Van Breusegem F, Kushnir S, Slooten L, Bauw G, Botterman J, Van Montagu M, et al. Processing of a chimeric protein in chloroplasts is different in transgenic maize and tobacco plants. Plant Mol Biol 1998; 38: 491-6.

[6] Kim EH, Suh SC, Park BS, Shin KS, Kweon SJ, Han EJ, et al. Chloroplast-targeted expression of synthetic cry1Ac in transgenic rice as an alternative strategy for increased pest protection. Planta 2009; 230: 397-405.

[7] Chen X. Study on the Genetic Stability of Plantlets Regenerated from Tobacco Mesophyll Protoplast. Journal of Anhui Agricultural Sciences 1999; 27: 365-6.(In Chinese)

[8] Zhu CX, Song YZ, Yin GH, Wen FJ. Induction of RNA-mediated Multiple Virus Resistance to Potato virus Y, Tobacco mosaic virus and Cucumber mosaic virus. Journal of Phytopathology 2009; 157: 101-7.

[9] Wang D, Bai XL, Liu Q, Zhu YR, Bai YL, Wang Y. Chloroplast-targeted expression of human sTRAIL protein in transgenic tobacco. unpublished.

[10] Daniell H. Transgene containment by maternal inheritance: Effective or elusive? Proc Natl Acad Sci U S A 2007; 104: 6879-80.