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Full Length Research Paper

Expression of human soluble tumor necrosis factor (TNF)-related apoptosis-inducing ligand in transplastomic tobacco

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The soluble extracellular domain of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (*sTRAIL*) can, as the whole length *TRAIL* protein, bind with its receptors and specifically induce the apoptosis of cancer cells; therefore, it has been developed as a potential therapeutic agent for various cancer treatments. As it has become an attractive technology for foreign protein production, especially for production of biopharmaceuticals, chloroplast engineering was applied in this study to express human *sTRAIL* protein in tobacco. Two transplastomic lines were obtained. Southern blot showed that *sTRAIL* gene was inserted into the right site of the tobacco chloroplast genome. RT-PCR results also confirmed that the foreign gene is transcribed in both lines. However, western blot showed that only one line accumulated *sTRAIL* protein stably, while the other line lost the ability to accumulate this protein after several rounds of subcultures. The possible reason for this unexpected phenomenon is discussed.

Key words: Chloroplast transformation, *sTRAIL*, pharmaceutical protein, expression, tobacco chloroplast.

INTRODUCTION

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (*TRAIL*), a new member of the TNF superfamily, was discovered by Wiley et al. (1995). It is a type II transmembrane protein. Its extracellular domain, 114-281 amino acids of carboxylic terminal, is the soluble part of TRAIL (*sTRAIL*). *sTRAIL* is capable of inducing apoptosis in tumor cells but not in normal cells (Ashkenazi et al., 1999). As a result of the selective apoptotic activity, TRAIL is regarded as a promising anti-cancer therapy that is currently in phase II clinical trials (Holoch and Griffith, 2009). Recombinant human *TRAIL* (*rhTRAIL*) shows encouraging results on Hodgkin's lymphoma in phase Ia trial (Herbst et al., 2006) and also affected the non-Hodgkin's lymphoma in phase Ib trial (Yee et al., 2007). Therefore, production of *sTRAIL* by recombinant bio-technique in bacterial (Lin et al., 2007), yeast (Xu et al., 2003), animal (Walczak et al., 1999), and recently in plant cells, has been investigated.

Plants can be developed as efficient bioreactors to produce pharmaceutical proteins. By "molecular farming", as it is called, people can manufacture pharmaceutical proteins in large scales with low cost. Proteins accumulated in plants are easy for storage and transport, and most importantly without the danger of contamination by human pathogens (Daniell, 2006). With the development and improvement of chloroplast transformation, “molecular farming” is becoming more and more attractive and prospective. Some plant-derived pharmaceutical proteins have been under-going clinical trials (http://www.molecularfarming.com). Chloroplast transformation provides a lot of advantages over traditional nuclear transformation, such as high level expression of foreign proteins (Oey et al., 2009) due to high polyploidy, absence of gene silence and position effects via homologous recombination (Daniell et al., 2002), multigene expression by polycistronic way (Zhou et al., 2007) and highly transgene containment due to chloroplast maternal inheritance (Ruf et al., 2007; Daniell, 2007). To date, many foreign genes have been expressed successfully in plant chloroplast and they showed

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biological activity, such as human somatotropin (hST) (Staub et al., 2000), human interferon gamma (INF-γ and INF-α2b) (Leelavathi and Reddy, 2003; Arlen et al., 2007) and proteins which are important in cancer therapy.

We have previously expressed *sTRAIL* in *Chlamydomonas reinhardtii*, in that it is a unicellular alga (Yang et al., 2006). Although *C. reinhardtii* has been successfully used for expression of several foreign proteins, the level of protein accumulated in its chloroplast is generally much lower than that in tobacco, which is the most widely used higher plant in protein expression with about 70% of total soluble proteins in some cases (Oey et al., 2009).

In this study, *sTRAIL* was expressed in tobacco chloroplast, and, to our knowledge, it is the first time to express this biopharmaceutical protein in a higher plant.

MATERIALS AND METHODS

Construction of transformation vector

Plasmid *pTRAIL* carrying *sTRAIL* gene was provided by Prof. Dexian Zheng (Chinese Academy of Medical Sciences and Peking Union Medical College, China). The sequence of *sTRAIL* gene was amplified from *pTRAIL* vector by PCR with primers pTup1 5´-CCCAAGCTTGATGGTGAGAGAAAGAGGTC-3´ and pTdn15´-ACGCGTCGACGTCTTAGCCAACTAAAAAGGCCC-3´, restriction sites of Hind III and Sal I are underlined. The 509 bp PCR product was cloned into PMD-19 simple T-vector (Takara, Dalian, China) for sequencing. The cloned *sTRAIL* fragment was excised by Hind III and Sal I from T-vector and ligated into 16APT (provided by Prof. Guifang Shen, Biotechnology Research Institute, Chinese Academy of Agricultural Sciences), which contains tobacco Prrn promoter, psbA terminator and Prrn-aadA-TpsbA expression cassette, to generate plasmid p16APT-sT. The part containing expression cassettes of aadA and *sTRAIL* gene was digested from 16APT-sT with *BamH I* and ligated into Bgl II site, between the homologous sequences rp12-trnH-psbA and trnK of pTRV (also provided by Prof. Guifang Shen), to construct chloroplast expression vector pTRV-sT.

Plant material and chloroplast transformation

Tobacco, *Nicotiana tabacum* L. cv. Petit Havana, kindly provided by Prof. Bock of Max Planck Institute of Molecular Plant Physiology, was grown in sterile conditions on MS medium (Murashige and Skoog, 1962) at 25°C under a 16 h light and 8 h dark cycle, and subcultured every 3 to 4 weeks. Young leaves were used for bombardment with DNA-coated 0.6 μm gold particles by PDS-1000/He Biolistic Particle Delivery System (Bio-Rad). After bombardment, leaves were cut into small pieces and cultured on RMOP (Zoubenko et al., 1994) medium containing 500 mg/L spectinomycin. Nine rounds of selection and regeneration were performed for homoplasmy. Positive shoots were rooted on MS medium containing 3% (w/v) sucrose, 0.6% (w/v) agar and 500 mg/L spectinomycin.

PCR analysis

The total DNA was extracted from spectinomycin-resistant plants and wild type tobacco with CTAB extraction buffer as described previously (Doyle and Doyle, 1990). PCR reaction was performed using primer pairs, specific for the foreign gene *sTRAIL* with Taq polymerase (TaKaRa, Dalian, China) according to standard procedure.

Southern blot

Plastid DNA was extracted as described previously (Gong et al., 1994). 3 μg DNA per sample digested with *BamH I* and *Not I* was separated on a 0.7% (w/v) agarose gel and transferred to Hybond-N+ nylon membranes (Amersham, Buckinghamshire, UK). DNA probe (0.6 kb), which is a part of trnK gene, was amplified by PCR with P3F (5´-TAAACAAGTAAAGACCCCTC-3´) and P3R (5´-TTAGTAAACCGGTTTGGTCC-3´). The probe was labeled with α-[32P]-dCTP (50 μCi) by using Random Primer DNA Labeling Kit (TaKaRa, Dalian, China). Hybridization was performed according to standard molecular cloning protocol (Sambrook and Russell, 2001).

Semi-quantitative RT-PCR analysis

Total RNA was extracted with TRNzol extraction buffer (TIANGEN Biotech., Beijing, China) following the protocol. cDNA synthesized by M-MLV (Promega) was used as template for PCR to amplify *sTRAIL* gene with specific primers. RNA without reverse transcription treatment was used as the template of the negative control. Primers NtrefF(5'-AGGAACCCAGAGGAGATT-3') and NtrefR (5'-ACAGTTGGGACTCGGAAA-3') were used for amplifying NtGAPDH (glyceraldehyde-phosphate dehydrogenase) gene which was used to calibrate the cDNA (accession no. AJ133422).

Western blot analysis

Total soluble proteins (TSP) from transformed and untransformed leaves (100 mg) were extracted following the method of Oey et al. (2009). Transplastomic lines from the 9th round regeneration and two rounds of shoot tip subculture after the 9th round regeneration were used for protein accumulation detection. Protein concentrations were determined with the Bradford assay according to the procedure of Sambrook and Russell (2001). However, 40 μg TSP per sample was separated on 15% SDS-containing polyacrylamide gels for 30 min at 100 V, and then for 3 h at 150 V. The separated proteins were transferred to a methanol-treated PVDF membrane (Millipore) by electro blotting at 100 mA for 1 h. The PVDF membrane was blocked with 5% milk in TBST buffer for 1 h at room temperature. The blocked membrane was incubated at room temperature in TBST solution containing a 1:1000 dilution of commercially available rabbit anti-*TRAIL* polyclonal antibodies (Sigma) for 2 h. The membrane was washed with TBST for 15 min four times, and then incubated with 1:5000 TBST dilution of horseradish peroxidase (HRP)-conjugated goat anti- rabbit IgG (Santa Cruz Biotechnology) for 1 h. After it was washed with TBST as previously described, the membrane was incubated with Pro-light HRP lighting substrate (TIANGEN Biotech., Beijing, China). Fluorescence signal was explored to X-ray films (FUJIFILM) and the hybridization band was confirmed by both pre-stained marker and western blot marker.

ELISA quantification of *sTRAIL*

Samples for ELISA analysis were prepared from wild type tobacco and transformed lines as described by Youm et al. (2010). Total soluble proteins were diluted with extraction buffer and quantified by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R & D system) according to the manufacturer's protocol.

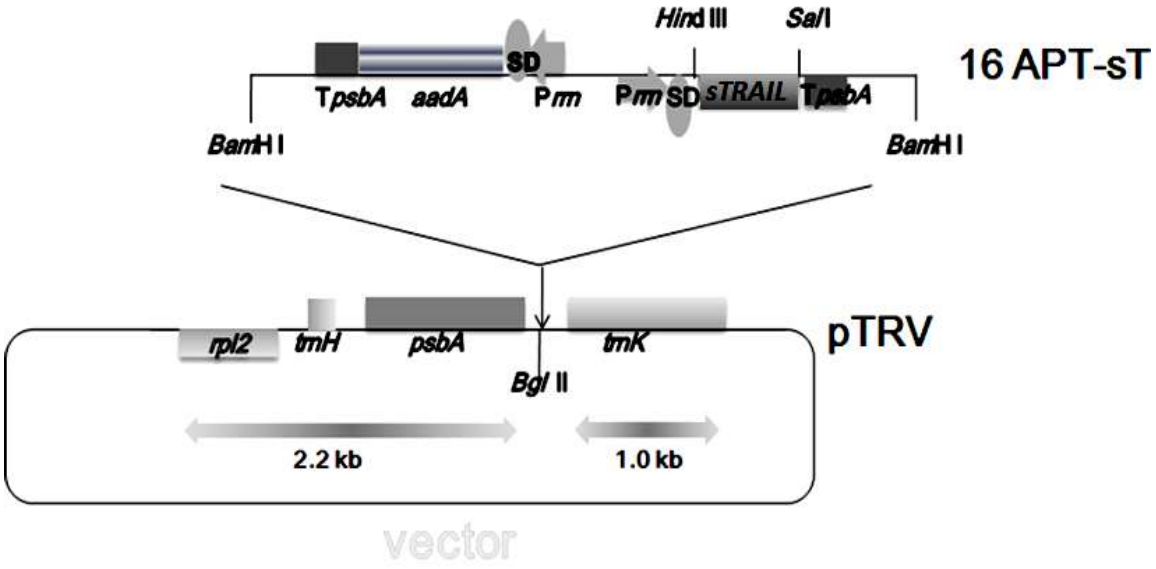

Figure 1. Construction of chloroplast transformation vector pTRV-sT.

RESULTS

Construction of chloroplast transformation vector

Chloroplast transformation vector was constructed as described in previously. As shown in Figure 1, *sTRAIL* encoding sequence was under the control of 16S rRNA strong promoter Prrn. It was seen that rbcL Shine-Dalgarno (SD) sequence and psbA terminator TpsbA were used for gene expression regulation (Shan et al., 1999), while aadA was used as a selection marker. Sequences of rpl2-trnH-psbA (2.2 kb) and trnK (1.0 kb) were used as flanking sequences (Zou et al., 1998) to target the expression cassette that will be integrated into chloroplast genome via homologous recombination.

Regeneration of transplastomic tobacco and DNA integration detection

After bombardment to the tobacco leaves with the chloroplast vector pTRV-sT and culture of leaf pieces on RMOP medium containing 500 mg/L spectinomycin, eight resistant lines were obtained. These lines were propagated and subjected to PCR-identification using *sTRAIL*-specific primers. The expected DNA fragment (509 bp) was amplified by PCR only from DNA samples of T3 and T8 lines (Figure 2), which indicated that these plants were transformed by *sTRAIL* encoding sequence.

For achieving the homoplasmy of transgene, nine ounds of regeneration were performed. DNA was then extracted from the transformed plants and wild type tobacco and was used for detecting the integration of *sTRAIL* encoding sequence in the chloroplast genome and homoplasmy of the transplastomic plants. The results (Figure 3a and b) showed that after digestion of the DNA by *BamH I* and *Not I* restriction enzymes, the predicted 2.7 kb DNA fragment was present in chloroplast genome DNA preparation from the transformed tobacco, which indicated that the *sTRAIL* encoding sequence was inserted into the right site of the tobacco chloroplast genome. In addition, the 4.8 kb fragment resulted from the digestion of wild type chloroplast genome DNA by *BamH I*, and was only detected in chloroplast genome DNA preparation from the wild type plants but not from T3 and T8 plants, which suggested that transplastomic lines all finished as homoplasmy after nine rounds selection and plant regeneration.

Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR analysis was carried out to test the transcript accumulation of *sTRAIL* gene. No DNA contamination was found in RNA samples from the transgenic plants (Figure 4d). As a result, these RNA samples were used in RT-PCR analysis with *sTRAIL* gene specific primers. GAPDH (glyceraldehydes-phosphate dehydrogenase) gene was used as a standard (Figure 4b) to calibrate the cDNA. The results showed that *sTRAIL* gene was transcribed in transplastomic lines and the transcription level was almost the same (Figure 4a).

Expression of *sTRAIL* protein

The accumulation of *sTRAIL* protein was analyzed by western blot. At the beginning of the 9th round homogenization, *sTRAIL* protein was detected in both

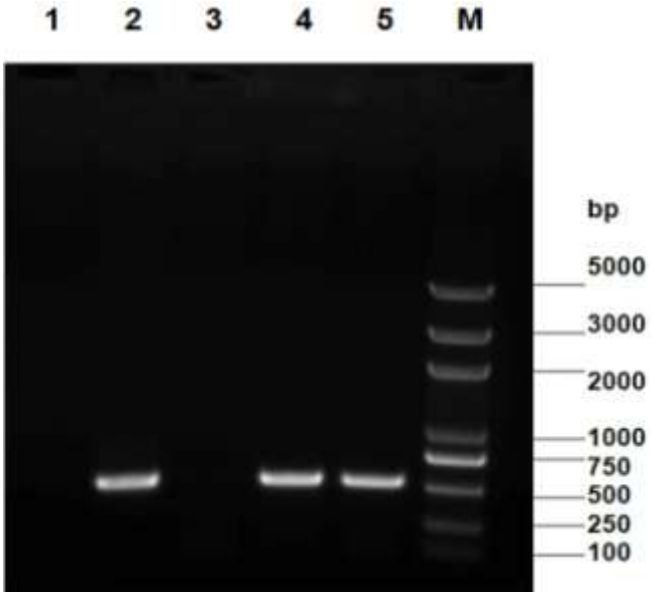

Figure 2. PCR analysis of *sTRAIL* insertion. Lane 1, Negative control using water as template; lane 2,positive control of pTRV-sT; lane 3, wild type tobacco; lanes 4 and 5, transformed tobacco lines T3 and T8; Lane M, marker trans 2K plus.

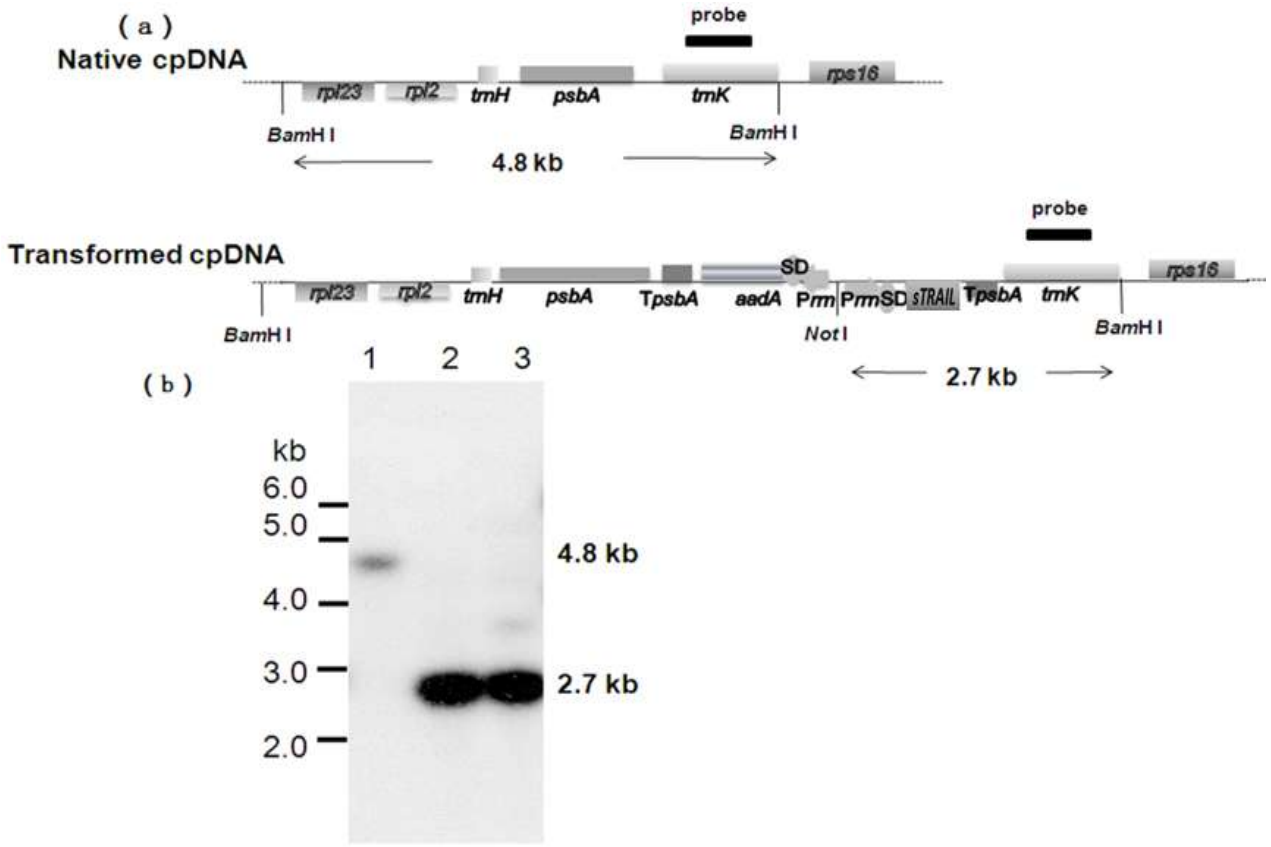

Figure 3. Southern blot analysis. (a) Scheme of the foreign gene integration in the chloroplast genome, in which a part of the *trnK* sequence (0.6 kb) was used as the hybridization probe represented with black bar; (b) result of the southern blot analysis. Lane 1, Wild type tobacco; Lanes 2 and 3, transgenic lines T3 and T8. Chloroplast genome DNA was digested together with *Bam*H I and *Not* I before separation by electrophoresis.

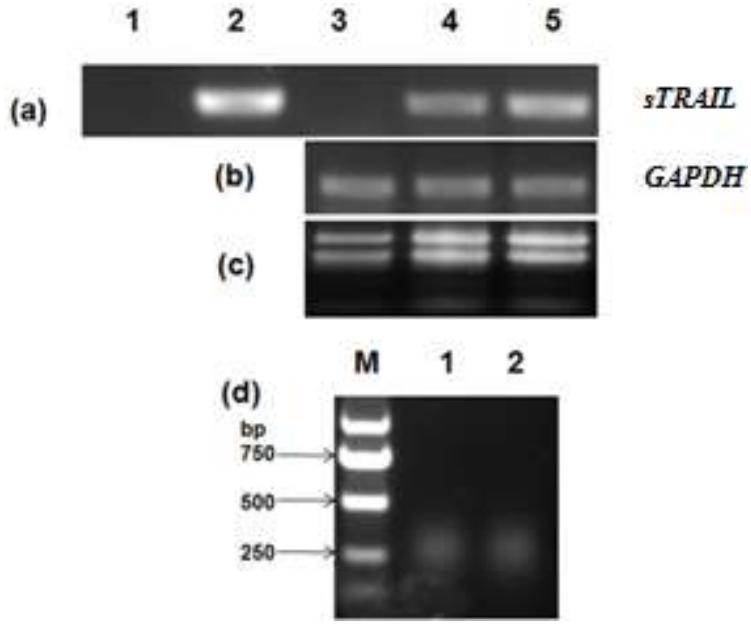

Figure 4. Semi-quantitative RT-PCR analysis of *sTRAIL* in transplastomic lines. (a) RT-PCR analysis. Lane 1, Negative control using water as template; Lane 2, positive control using pTRV-sT as template; Lane 3, wild type tobacco; Lanes 4 and 5, transgenic lines T3 and T8; (b) GAPDH gene used as reference gene for internal calibration; (c) The results of electrophoresis of total RNA; (d) PCR analysis using total RNA as template. Lanes 1 and 2,transgenic lines T3 and T8; Lane M, DNA marker trans2k plus.

transgenic lines (Figure 5a). However, it was surprisingly found that after two rounds of shoot-tip subculture of these homogenized plants, *sTRAIL* could only be detected in line T3, but not in T8 plants (Figure 5b). Interestingly, the accumulation of *sTRAIL* in T3 plants increased obviously. The ELISA assay showed that *sTRAIL* protein in the TSP of these T3 plants was about 0.9% (Figure 5c).

DISCUSSION

In this study, *sTRAIL* protein was successfully expressed in tobacco chloroplast and the highest accumulation level was estimated as about 0.9% of TSP, which is about 30 folds higher than that expressed via the targeting expression way with chloroplast transit peptide (Wang et al., 2010). The expression level was also generally higher than that accumulated in transgenic *Chlamydomonas* (Yang et al., 2006).

Two different transplastomic lines were obtained in this study, and both showed efficient transcription of *sTRAIL* (Figure 4). However, T8 plants failed to produce *sTRAIL* protein after subculture of the shoot tips compared with T3 plant (Figures 5a and b). From the semi-quantitative RT-PCR analysis, it was seen that the transcription level of *sTRAIL* in two transplastomic lines was almost the same. One possible reason could be that in T8 plants, the translation of *sTRAIL* protein was poorly affected. It is well known that SD sequence is a key element for protein translation both in prokaryotes and in chloroplasts of plants. In this study, the SD sequence of rbcL gene was constructed at an upstream of *sTRAIL*. This SD sequence has been reported to be efficient for controlling protein translation (Svab and Maliga, 1993; Machin et al., 2004) and the efficiency was also verified in the study's T3 plants. Nevertheless, the engineered SD sequence in T8 plants might have gotten a modification during the subculture. For example, it might have had a spontaneous mutation within the sequence. It is also possible that this sequence changed through recombination with another SD within the native tobacco chloroplast genome. Tobacco chloroplast genome possesses about 122 genes (Shinozaki et al., 1986) and the SD sequences for these genes are not identical, rather their translation efficiency is different. Recombination of sequences within insertions of transformed chloroplast with native sequences was previously reported by Staub and Maliga (1992) and Iamtham and Day (2000). However, the mechanisms underlying it are not clear and should be intensively investigated in the future.

It was observed that the T3 and T8 plants had different phenotypes (Figure 6). Leaves of T3 plants are pale green, chlorotic and biomass-reduced, while T8 plants

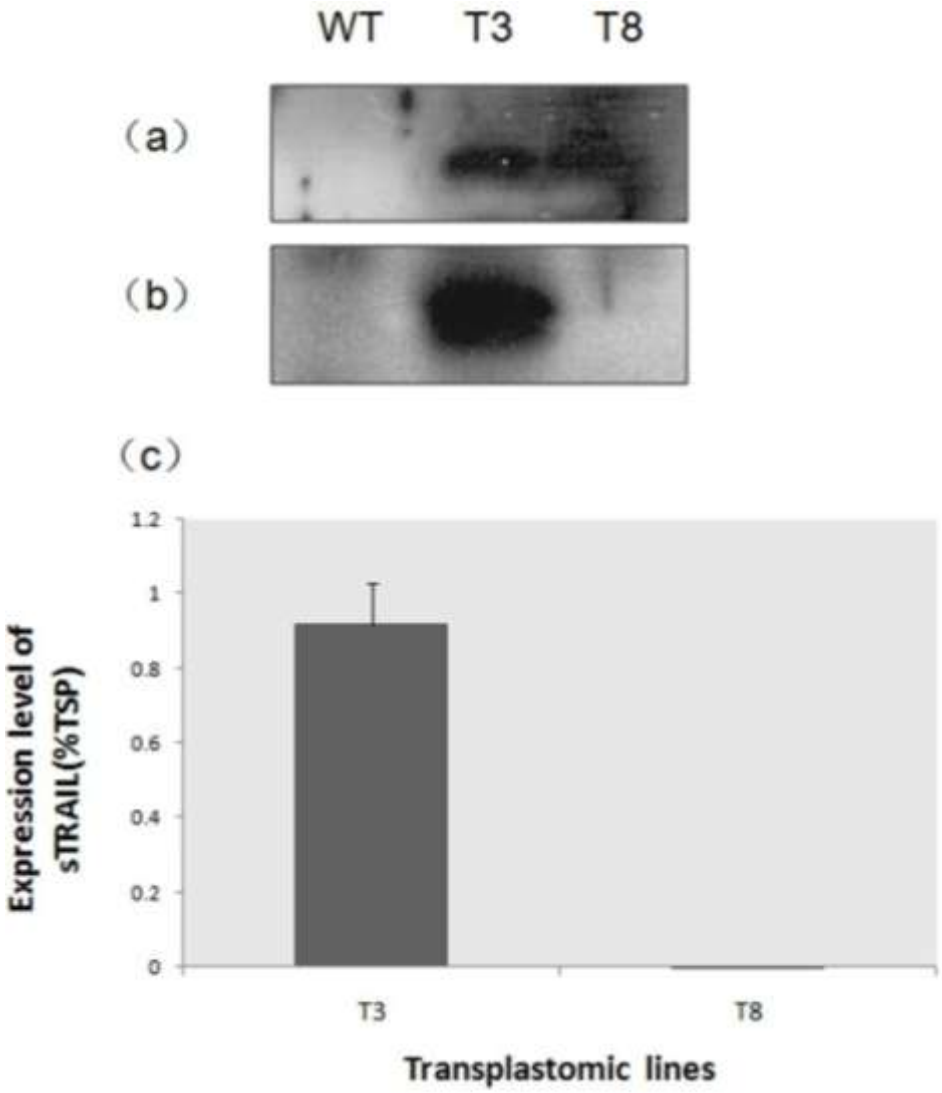

Figure 5. *sTRAIL* protein expression analysis. (a) and (b) Western blot analysis of *sTRAIL* protein expression in transgenic lines at early stage of the 9th round regeneration and after two rounds of shoot tip subculture of the 9th round regenerated plants; Lane WT, wild type plant; (c) ELISA assay of *sTRAIL* protein expressed in transplastomic lines after two rounds of shoot tip subculture of the 9th round regenerated plants. Values are averages of three experiments with standard deviations.

looked almost like the wild type except for some small pale green sectors. In animals, it has been confirmed that *sTRAIL* induces the death of cancer cells (Ashkenazi et al., 1999). Therefore, *sTRAIL* protein might also be toxic to plants, and its accumulation within chloroplast could affect the physiology of plants severely. The accumulation of recombinant protein can bring a toxic effect (Tregoning et al., 2003; Hennig et al., 2007; Scotti et al., 2009). If this is the case, the expression of *sTRAIL* in other genotypes of tobacco may be an alternative way to eliminate such effect and increase the protein accumulation (McCabe et al., 2008). Furthermore, there might be a need to try other higher plants, in a larger range, such as Arabidopsis thaliana, Oryza sativa, Solanum tuberosum, Medicago truncatula, Lactuca saiva and Glycine max.

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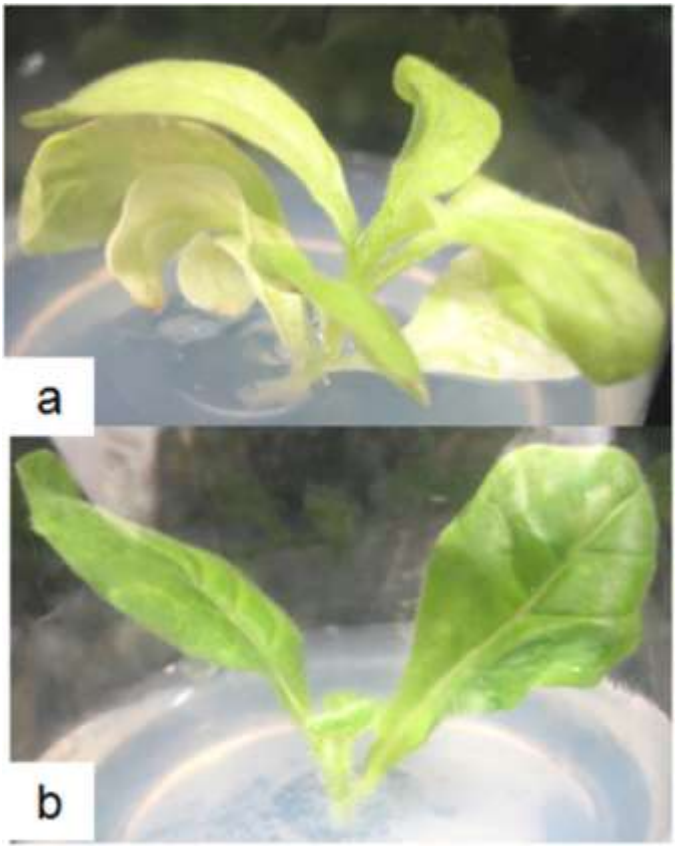

Figure 6. Phenotype of the transplastomic tobacco line T3 (a) and line T8 (b).

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