High-density seedling expression system for the production of bioactive human cardiotrophin-1, a potential therapeutic cytokine, in transgenic tobacco chloroplasts

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Summary

Histidine-tagged human cardiotrophin-1 (hCT-1), a recently discovered cytokine with excellent therapeutic potential, was expressed in tobacco chloroplasts under the transcriptional and translational control of two different promoters (*rrn* and *psbA*) and 5′-untranslated regions (5′-UTRs) (*psbA* and phage T7 *gene 10*). The *psbA* 5′-UTR promotes recombinant hCT-1 (rhCT-1) accumulation in chloroplasts at higher levels (eight-fold) than those obtained for the phage T7 *gene 10* 5′-UTR, regardless of the promoter used, indicating that the correct choice of translational control element is most important for protein production in chloroplasts. The maximum level of rhCT-1 achieved was 1.14 mg/g fresh weight (equivalent to 5% of total soluble protein) with the *psbA* promoter and 5′-UTR in young leaves harvested after 32 h of continuous light, although the bioactivity was significantly lower (~35%) than that of commercial hCT-1. However, harvesting in the dark or after 12 h of light did not result in a significant decrease in the bioactivity of rhCT-1, suggesting that 32 h of over-lighting affects the biological activity of rhCT-1. Because high levels of rhCT-1 accumulation took place mainly in young leaves, it is proposed that seedlings should be used in a 'closed system' unit, yielding up to 3.2 kg per year of rhCT-1. This amount would be sufficient to meet the estimated annual worldwide needs of hCT-1 for liver transplantation surgery in a cost-effective manner. Furthermore, our strategy is an environmentally friendly method for the production of plant-based biopharmaceuticals.

Introduction

New discoveries involving therapeutic proteins offer exciting possibilities for the treatment of human pathologies. Several challenges still exist in the production of these therapeutic proteins. They must be obtained on a cost-effective basis in sufficient amounts to meet current growing needs. Plant-made pharmaceuticals are becoming a major commercial development in biotechnology (Ma *et al.*, 2005). They offer significant advantages over other production systems, such as low production costs, easy scale-up, absence of human pathogens and convenient storage and processing. Although the plant nuclear genome has been used to express human therapeutic proteins for more than a decade (Fischer *et al.*, 2004), expression levels have been disappointingly low, usually far lower than 1% of total soluble protein (TSP), a yield that is estimated by industry to be sufficient for the production of biopharmaceuticals in a cost-effective manner (Farran *et al.*, 2002).

The chloroplast transgenic approach has been developed as an alternative to nuclear expression (Daniell, 2006; Bock, 2007). The accumulation of exceptionally large quantities of

foreign proteins (De Cosa *et al*., 2001; Molina *et al*., 2004) has been achieved partly as a result of high transgene polyploidy (up to 20 000 copies/cell) (Maliga, 2004). Human therapeutic proteins, such as somatotropin (Staub *et al*., 2000), serum albumin (Fernández-San Millán *et al*., 2003), various interferon types (Leelavathi and Reddy, 2003; Arlen *et al*., 2007) and proinsulin (Ruhlman *et al*., 2007), have been produced at high levels (6%–20% of TSP). However, other attempts have failed (Magee *et al*., 2004; Wirth *et al*., 2006), indicating that post-transcriptional mechanisms are likely to be involved. Chloroplast-derived therapeutic proteins have also shown correct folding, disulphide bond formation and functionality (Daniell, 2006). Other advantages include a much more stable and uniform expression among transgenic lines, the absence of epigenetic effects and gene silencing, the ability to simultaneously express multiple transgenes by linking them together in operons (Quesada-Vargas *et al*., 2005), an increase in transgene containment as a result of the maternal inheritance of plastids in most crop plants (Hagemann, 2004), and the expression of foreign proteins lacking methionine as the N-terminal amino acid (Fernández-San Millán *et al*., 2007).

Cardiotrophin-1 (CT-1) is a member of the interleukin-6 family, which is structurally and biologically related to cytokines. It was first identified by its ability to induce hypertrophic response in cardiac myocytes (Pennica *et al*., 1995). CT-1 induces its biological effects through the shared signalling subunit gp130 (Pennica *et al*., 1996a), and stimulates the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway to transmit hypertrophic and cytoprotective signals (Robledo *et al*., 1997). It has been reported that recombinant CT-1 (rCT-1) may defend cardiac cells against hypoxic damage (Brar *et al*., 2001), hepatocytes against acute liver injury (Bustos *et al*., 2003) and neurones against oxidative injury (Wen *et al*., 2005). Recently, CT-1 has been defined as a natural defence of the liver against apoptosis (Marques *et al*., 2007) and ischaemia–reperfusion injury, and is a key mediator of the protective effect induced by ischaemic preconditioning (Iñiguez *et al*., 2006). From all these findings, it is conceivable that this cytokine may offer a high therapeutic potential. Other valuable therapeutic uses of CT-1 are in the treatment of spinal muscular atrophy (Lesbordes *et al*., 2003) and in surgery for liver cancer or transplantation (Bustos *et al*., 2003). Unfortunately, the only human rCT-1 (rhCT-1) commercially available is produced in *Escherichia coli* at a cost of about $5000/mg. As a consequence, no *in vivo* assays have been performed with the high-cost rhCT-1 to date. To our knowledge, no specific literature concerning recombinant expression systems for

hCT-1 protein has been reported. Pennica *et al*. (1996b) referred to the production of hCT-1 in human 293 cells as part of their experimental procedure, but no production data were given. Alternative strategies are therefore required to reduce costs to facilitate potential therapeutic application.

In this study, the production of rhCT-1 in tobacco chloroplasts is pursued. The hCT-1 protein contains 201 amino acids, two cysteine residues (no experimental evidence of disulphide bridge linkage has been reported) and lacks potential N-linked glycosylation sites (Pennica *et al*., 1996b). It is therefore a good candidate for chloroplast expression. The hCT-1 was generated as a recombinant protein with N-terminal fusion of a histidine (His) tag and enterokinase cleavage site. The optimization of the yield of recombinant protein in transplastomic plants is a key factor for cost-effective production. In this context, the effectiveness of different 5′-regulatory sequences, leaf age and growing conditions on the expression level and bioactivity of the protein were analysed. High yields of rhCT-1 were achieved by harvesting young tobacco leaves grown in a confined environment. For the first time, it was found that the hCT-1 bioactivity level produced in chloroplasts can be down-regulated by over-lighting.

Results and discussion

Chimeric rhCT-1 genes and vector construction

To achieve high levels of rhCT-1 protein accumulation in plastids, two chimeric genes were initially tested, each of which carried the 3′-mRNA stability elements from the chloroplast ribosome small subunit 16 gene (T*rps16*) (Staub and Maliga, 1994). To optimize hCT-1 protein accumulation in transgenic chloroplasts, different 5′-regulatory sequences were used. In the plasmid pL3-PrrnG10L-CT1 (Figure 1a), the *rhct1* gene was expressed from the plastid constitutive promoter of the rRNA operon (P*rrn*) fused to the leader sequence of the phage T7 *gene 10* (*G10*L). High levels of foreign protein expression in chloroplasts (7%–25% of TSP) have been obtained for different proteins using this 5′-sequence (Staub *et al*., 2000; Tregoning *et al*., 2003). In the pL3-PpsbA-CT1 vector (Figure 1a), the tobacco promoter and 5′-untranslated region (5′-UTR) of the *psbA* gene were used. Foreign genes under the control of the *psbA* promoter and its 5′-UTR are expressed at very high levels (Leelavathi and Reddy, 2003; Molina *et al*., 2004; Arlen *et al*., 2007; Fernández-San Millán *et al*., 2008).

The chimeric genes were cloned into chloroplast transformation vector pL3, developed in this laboratory (Figure 1b). This vector integrates genes of interest into the plastid
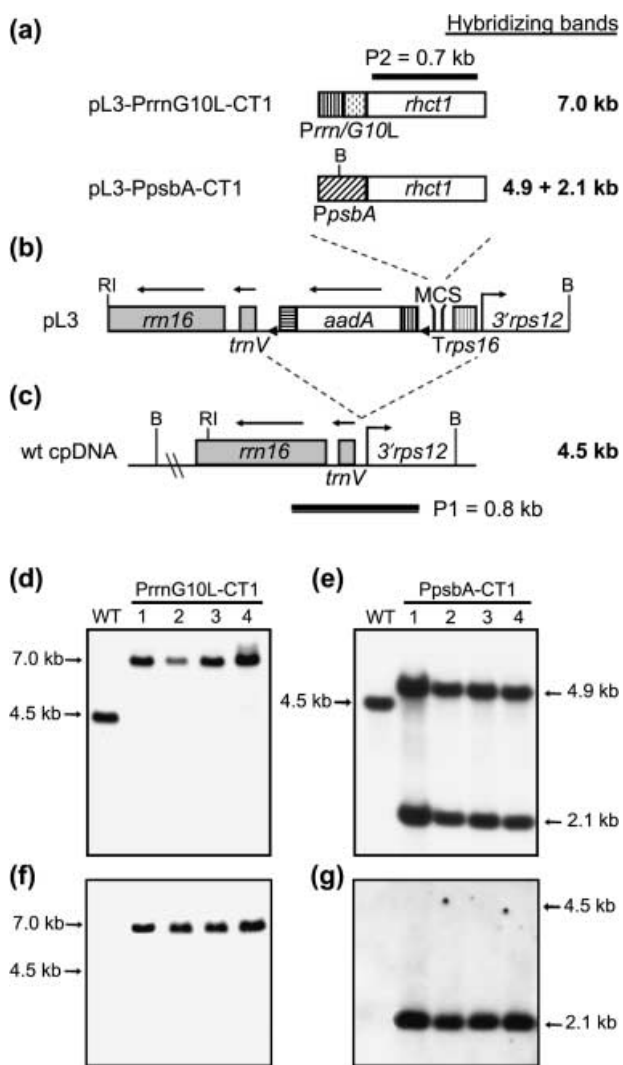
Figure 1 Integration of chimeric *rhct1* genes into the chloroplast genome and homoplasmy verification. (a) *rhct1* chimeric genes are driven by plastid 5′-expression signals, P*psbA* or P*rrn/G10*L, and the T*rps16* trailer needed for mRNA stability. The P2 probe comprised the human cardiotrophin-1 (*rhct1*) coding region. The numbers to the right indicate the predicted hybridizing bands when digested with *Bgl*II and probed with P1. (b) Chimeric genes are cloned next to the *aadA* expression cassette, which is flanked by the *loxP* sequences (black triangles), in the pL3 plastid transformation vector. The arrows above the boxes show the direction of transcription. (c) Chloroplast genomic sequence at the insertion site between the *trnV* and 3′*rps12* genes. The 0.8-kb fragment (P1) of the targeting region for homologous recombination was used as a probe for Southern blot analysis. (d–g) Southern blot analysis of four independent lines (1–4) for each chimeric transgene: P*rrn/G10*L-*rhct1*-T*rps16* (d, f) and P*psabA*-*rhct1*-T*rps16* (e, g) using the P1 probe (d, e) or the *rhct1* coding region P2 probe (f, g). B, *Bgl*II; MCS, multiple cloning sites; RI, *Eco*RI; WT, wild-type Petit Havana plant.

genome between the *trnV* and 3′*rps12* genes in the inverted repeat region. These targeting sequences have been widely used in chloroplast transformation and have not exhibited any pleiotropic effect (Maliga, 2004). The chimeric *aadA*

gene, which confers resistance to spectinomycin and streptomycin, was driven by the P*rrn* promoter and the *psbA* terminator. This *aadA* cassette was flanked by *loxP* sites, which permit the complete excision of the antibiotic resistance gene after transformation (Lutz and Maliga, 2007). Because the protein synthetic machinery of chloroplasts is similar to that of *E. coli*, rhCT-1 expression of the pL3-PrrnG10L-CT1 and pL3-PpsbA-CT1 vectors was initially tested in *E. coli*. Western blot analysis of sonicated *E. coli* cell extracts showed the presence of a 23-kDa rhCT-1, similar to commercially available hCT-1 (data not shown).

The vectors were bombarded into tobacco leaves as described previously (Daniell, 1997). After 5 weeks, several primary shoots appeared from each bombarded leaf as a result of independent transformation events. Putative transformed shoots were identified by growth on 500 mg/L spectinomycin, and were then submitted to consecutive rounds of regeneration in this selective medium.

Chloroplast integration and homoplasmy verification

The stable integration of foreign genes into the chloroplast genome and homoplasmy of the transformed plants were confirmed by Southern blot analysis. After three rounds of selection, total leaf DNA from regenerated plants was digested with the *Bgl*II restriction enzyme, which cuts two positions flanking the insertion site outside the left and right recombination regions (Figure 1c) and one position in the *psbA* promoter (Figure 1a). The 0.8-kb probe P1, which is homologous to the flanking regions *rrn16/trnV* and 3′*rps12* (Figure 1c), was used. Thus, wild-type plastomes release a 4.5-kb DNA fragment, whereas transformed plastomes release either a 7.0-kb DNA fragment (for PrrnG10L-CT1 plants) or two DNA fragments of 4.9 and 2.1 kb (for PpsbA-CT1 plants). Four independent lines transformed with each of the transformation vectors were analysed (Figure 1d–g). The absence of a 4.5-kb fragment in transgenic lines indicates homoplasmy (Figure 1d, e). When the blot was stripped and reprobed with the P2 hCT-1 coding region probe (Figure 1a), hybridization was only detected in the transplastomic lines, as expected (Figure 1f, g). Southern analysis of the T_1 generation confirmed that all eight transgenic lines analysed maintained homoplasmy (data not shown).

Effect of 5′-regulatory sequences on the expression of rhCT-1 in tobacco chloroplasts

T_1 generation plants were analysed for levels of rhCT-1 accumulation in leaves. Sodium dodecylsulphate-polyacrylamide
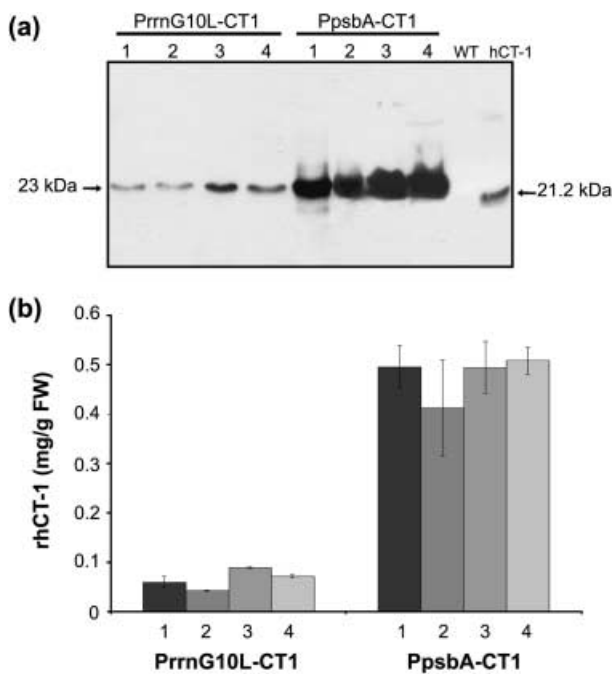
Figure 2 Accumulation of recombinant human cardiotrophin-1 (rhCT-1) protein in tobacco leaves. Four independent lines (1–4) for each construct (PrrnG10L-CT1 and PpsbA-CT1) were analysed. (a) Western blot analysis of rhCT-1. Ten micrograms of total soluble protein were loaded per lane. hCT-1, 75 ng of commercial human cardiotrophin-1 (PeproTech); WT, wild-type Petit Havana plant. (b) Quantification of rhCT-1 expression in transplastomic plants by enzyme-linked immunosorbent assay (ELISA). Total soluble protein was extracted from young, fully expanded leaves of potted plants in the walk-in room harvested 40 days after transplantation. Expressions levels are indicated as milligrams of rhCT-1 per gram leaf fresh weight (FW). The data are presented as the mean ± standard error of measurements on three individual plants per line.

gel electrophoresis (SDS-PAGE) and Western blot analysis were performed. Monomers of rhCT-1 protein were detected at approximately 23 kDa (Figure 2a), which is slightly larger than the size of commercial hCT-1 (21.2 kDa) as a result of the polyhistidine tag and enterokinase cleavage site in our construct. The signal detected in the Western blot was specific for rhCT-1 because no band was present in the

wild-type plant (Figure 2a). Plants transformed with the vector pL3-PpsbA-CT1 accumulated much higher levels of recombinant protein than did plants transformed with pL3-PrrnG10L-CT1 (Figure 2a). This finding is in agreement with results obtained in *E. coli*, where higher levels of rhCT-1 were found in cell extracts transformed with pL3-PpsbA-CT1 (data not shown).

The levels of recombinant hCT-1 expression in young fully expanded leaves of plants 40 days after transplantation were determined using an enzyme-linked immunosorbent assay (ELISA). Large differences in the accumulation of rhCT-1 were observed between plants transformed with the two different vectors (Figure 2b), confirming the previous Western blot results. The transplastomic PrrnG10L-CT1 lines accumulated rhCT-1 in leaves at levels ranging from 0.042 to 0.090 mg/g fresh weight (FW), whereas leaves of PpsbA-CT1 lines produced high levels of recombinant protein, ranging from 0.41 to 0.51 mg/g FW (Figure 2b). On average, this level of expression is about eight-fold higher than that of the PrrnG10L-CT1 lines. Although very high levels of recombinant protein have been achieved using *PrrnG10L* (Staub *et al.*, 2000; Tregoning *et al.*, 2003), very low levels (Ye *et al.*, 2001) or levels lower than those obtained with *psbA* 5′-UTR (Dhingra *et al.*, 2004; Ruhlman *et al.*, 2007) have also been reported. An enhanced translation governed by the *G10L* 5′-UTR could be gene specific, being critical not only the sequences downstream of the translation initiation codon (Herz *et al.*, 2005), but also each individual mRNA (Kuroda and Maliga, 2001).

Northern blot analysis was performed to determine whether or not changes in *rhct1* mRNA accumulation could account for the observed changes in plastid rhCT-1 accumulation. Total cellular RNA extracted from the same leaf samples as used for rhCT-1 quantification was employed for Northern blot analysis. The *rhct1* coding region probe showed that transcripts from the *psbA* promoter were more abundant than those from the *rrn* promoter in the transplastomic lines (Figure 3, top). Similar findings were obtained by

Figure 3 Steady-state levels of *rhct1* mRNA in the transplastomic leaves. Total RNA was extracted from young, fully expanded leaves of potted plants in the walk-in room harvested 40 days after transplantation. Seven plants for each construct (PrrnG10L-CT1 and PpsbA-CT1) were analysed by Northern blot. An aliquot of 5 μg of total cellular RNA was loaded per lane. Blots were probed for human cardiotrophin-1 (hCT-1) (top) and 16S rRNA (bottom, loading control). WT, wild-type Petit Havana plant.
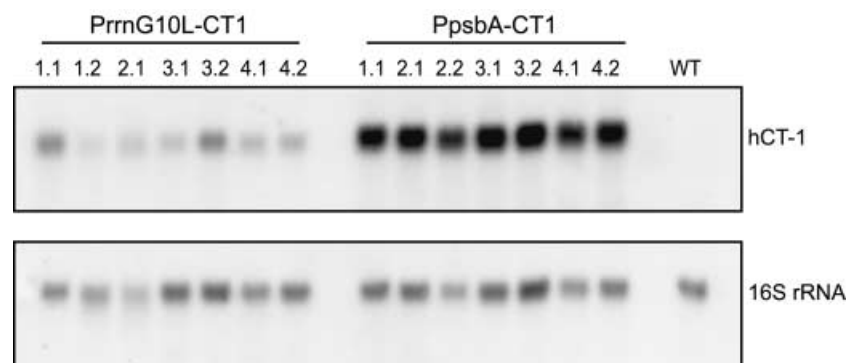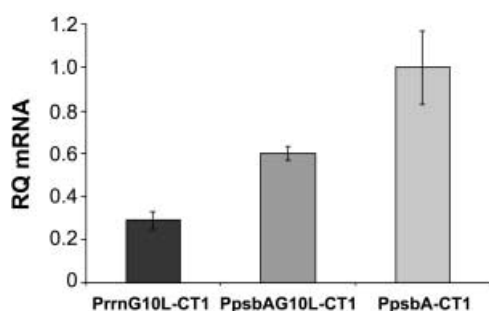
Figure 4 Relative amount of *rhct1* mRNA in leaves of transplastomic plants measured by quantitative real-time polymerase chain reaction. Data are normalized to the plastid *aadA* transcript and computed relative to the PpsbA-CT1 value, which was set to unity. Data are presented as the mean ± standard error of measurements from 9–12 samples (three to four independent plants per three biological replicates) per construct.

Dhingra *et al.* (2004). To rule out any potential loading artefact, the Northern blot was stripped and reprobed with a 16S rRNA-encoding DNA fragment (Figure 3, bottom). In this study, the chimeric genes have the same coding region (*rhct1*) and 3′-UTR (T*rps16*), but differ in the promoter (P*psbA* and P*rrn*) and 5′-UTR (*psbA* and *G10*L). Therefore, the increase in mRNA levels may be caused by either an increase in transcription rate or mRNA stability because of the leader sequence.

To study the significance of a putative interaction between promoter and 5′-UTR and how this could affect rhCT-1 accumulation in chloroplasts, a new transplastomic plant was constructed expressing *rhct1* under the control of a chimeric promoter that combines the *psbA* promoter with the *G10*L 5′-UTR. This chimeric gene was cloned into the chloroplast transformation vector pL3, and bombarded into tobacco leaves as described above, to obtain homoplasmic PpsbAG10L-CT1 plants (Figure S1, see 'Supplementary material'). Real-time polymerase chain reaction (PCR) was performed to quantify the *rhct1* mRNA accumulated in these plants, and was compared with that produced by PpsbA-CT1 and PrrnG10L-CT1 plants. Real-time PCR results indicated that *rhct1* transcripts of PpsbA-CT1 plants were 1.7-fold more abundant than those of PpsbAG10L-CT1 plants (Figure 4). Although these plants expressed rhCT-1 under the control of the same promoter (*psbA*), the mRNA increase could be explained by a greater stability of the transgene transcript conferred by the *psbA* 5′-UTR. Several authors have already reported a high stability of *psbA* transcripts in mature leaves (Kim *et al.*, 1993; Zoschke *et al.*, 2007), and the importance of the *psbA* 5′-UTR for mRNA stabilization and translation efficiency (Zou *et al.*, 2003). Likewise, *rhct1* transcripts from P*psbAG10*L were two-fold more abundant than those from P*rrnG10*L (Figure 4). In this case, both transcripts share the

*G10*L 5′-UTR, and therefore their mRNA stability should be identical. Thus, the observed mRNA differences could only be attributed to the promoter strength. Several authors have reported that 16S rRNA has a higher transcription rate than *psbA* early in chloroplast development, but lower in mature chloroplasts (Baumgartner *et al.*, 1993; DuBell and Mullet, 1995). This change is consistent with the protein synthesis mechanisms of chloroplasts. Thus, although the synthesis of the plastid decoding apparatus occurs early in chloroplast development, the ability of this machinery to synthesize photosynthetic proteins takes place in the mature chloroplast. Recently, Zoschke *et al.* (2007) have also shown that *psbA* transcription rates in young and mature leaves of *Arabidopsis* plants are two- to three-fold higher than those of the *rrn16* gene. Therefore, it is concluded that, under our conditions, the *psbA* promoter is stronger than the *rrn* promoter, and the *psbA* 5′-UTR confers more stability than the *G10*L 5′-UTR to the transgene transcript.

The protein accumulated in PpsbAG10L-CT1 plants (0.07 ± 0.01 mg/g FW) matched that of PrrnG10L-CT1 plants (Figure 2b), and both were much lower than that in PpsbA-CT1 plants (Figure 2b). Thus, protein levels seemed to be influenced more by the 5′-UTR than by mRNA abundance. Such a lack of correlation between transcript abundance and protein accumulation has been reported previously when the *psbA* 5′-UTR was used (Staub and Maliga, 1994; Fernández-San Millán *et al.*, 2003), suggesting an important role of the *psbA* 5′-UTR in the enhancement of translation (Eibl *et al.*, 1999). Recently, Yukawa *et al.* (2007) have shown that the 5′-UTR of the *psbA* gene results in the most efficient translation rate of 11 tobacco plastid genes studied. In this work, it was clearly demonstrated that the relative *rhct1* translation efficiency of the *psbA* 5′-UTR was much higher than that of the *G10*L 5′-UTR. In addition, it was shown that the translatability of the mRNA, determined by the 5′-translation control elements, was more important for chloroplast protein expression than mRNA abundance. Indeed, the choice of 5′-UTR may affect protein yields over a 10 000-fold range (Maliga, 2003). It is thus advisable, in order to increase protein yields, to test different 5′-UTR sequences before proceeding with the expression of a new transgene in the chloroplast.

Characterization of rhCT-1 in transgenic plants

The external appearance, age and environmental factors, such as lighting and temperature, contribute to the levels of expressed protein (Stevens *et al.*, 2000; Arlen *et al.*, 2007). As mentioned above, two different 5′-UTRs were used in this study to enhance the translation of *rhct1* cDNA. The T7 *G10*L

5'-UTR appears to facilitate constitutive translation of the transgene (Kumar *et al.*, 2004), whereas the translation of *psbA* 5'-UTR is regulated by light in tobacco plastids (Staub and Maliga, 1994). First, the changes in rhCT-1 levels were examined under different light regimes, comparing the expression levels in transplastomic plants (previously grown under a 16-h light/8-h dark cycle) when placed for 20 h in darkness or 12, 24, 32 and 50 h in continuous light. No significant changes in rhCT-1 level were observed between darkness and 12 h of light (Figure 5a), suggesting that the decrease in translation efficiency by *psbA* 5'-UTR in darkness
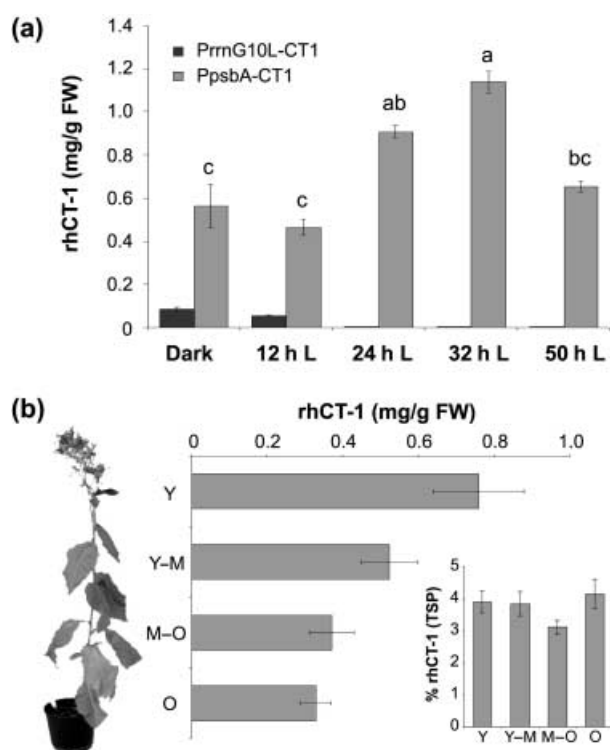
(Trebitsh *et al.*, 2000) may be counterbalanced by an increase in protein stability under these conditions (Wirth *et al.*, 2006). Under continuous light, the levels of rhCT-1 accumulated in the leaves of PrrnG10L-CT1 plants were below the detection limit of ELISA (2 μg/g FW). Nevertheless, rhCT-1 produced in leaves of PpsbA-CT1 plants accumulated as the continuous light exposure increased, reaching a peak after 32 h of continuous illumination (Figure 5a), with a yield of 1.14 mg/g FW, equivalent to 4.8% of TSP. Several studies have demonstrated that the *psbA* 5'-UTR confers light-dependent accumulation to other foreign proteins (Fernández-San Millán *et al.*, 2003; Dhingra *et al.*, 2004; Watson *et al.*, 2004; Arlen *et al.*, 2007). Notwithstanding this, the levels of rhCT-1 decreased after 50 h of continuous light exposure (Figure 5a). Similar observations have been reported by other authors (Dhingra *et al.*, 2004; Arlen *et al.*, 2007). The rhCT-1 decrease detected after 50 h of continuous light could reflect either a reduction in protein synthesis or a higher protein turnover induced by over-lighting conditions (Zheng *et al.*, 2002).

To examine the variation in rhCT-1 levels in differently developed leaves, TSP extracts from the highest expressing transgenic PpsbA-CT1 plant were prepared from leaves harvested at different positions along the plant, and subjected to ELISA. The relative amount of rhCT-1 protein per gram FW was highest in the youngest leaves (Figure 5b). Old senescent leaves showed much smaller amounts of recombinant protein, about 50% of that in young leaves (Figure 5b). The content of TSP also decreased from 23 mg/g FW in young leaves to 9 mg/g FW in old leaves, and the profiles of recombinant protein matched those of TSP. The amount of rhCT-1 expressed as a percentage of TSP remained basically unchanged (Figure 5b), as described previously (Stevens *et al.*, 2000; Molina *et al.*, 2004). The lower content of recombinant protein in older leaves of transgenic plants is not surprising because senescence activates proteolytic activity (Sakamoto, 2006), which degrades both endogenous and foreign proteins. However, several studies have reported that optimal levels of expression can be achieved in mature and old leaves of fully grown plants (Staub *et al.*, 2000; De Cosa *et al.*, 2001; Fernández-San Millán *et al.*, 2003). In these reports, the recombinant protein formed inclusion bodies (Fernández-San Millán *et al.*, 2003), folded into stable crystalline structures (De Cosa *et al.*, 2001) or aggregated via a chaperonin-like effect of ubiquitin (Staub *et al.*, 2000), protecting them from degradation. When a protein more susceptible to degradation is expressed in plastids, the highest yields are obtained in fast-growing, young leaves, whereas no protein or very low levels are present in mature leaves (Birch-Machin *et al.*, 2004; Herz *et al.*, 2005).

Figure 5 Characterization of transgenic recombinant human cardiotrophin-1 (rhCT-1)-producing plants. (a) Study after different hours of lighting. Samples of leaves were collected from potted plants transformed with PrrnG10L-CT1 or PpsbA-CT1 after a 20-h dark period or at the indicated hours of light (L). Expressions levels are indicated as milligrams of rhCT-1 per gram fresh weight (FW). Data are presented as the means ± standard error of measurements on four individual plants per construct. Different letters indicate statistically different values at $P = 0.05$ using the Duncan test. (b) rhCT-1 accumulation in leaves of potted plants in the walk-in room at different developmental stages. The plant to the left indicates the respective positions of the different leaves (Y, Y–M, M–O, O) in the plant. Total soluble protein was extracted from PpsbA-CT1 transformed plants and quantified by enzyme-linked immunosorbent assay (ELISA). Expressions levels are indicated as milligrams of rhCT-1 per gram FW, and as percentage of total soluble protein (TSP) (bottom right-hand corner). The data are presented as the mean ± standard error of measurements on four individual plants. Y, young leaves; M, mature leaves; O, old leaves.

The expression level of recombinant protein is thus highly dependent on tissue type, plant age and culture conditions. For the assessment of cost-effective protein production in plant plastids, all of these factors should be taken into account. In our work, higher levels of recombinant accumulation were achieved in fast-growing, young leaves of fully grown plants harvested after 32 h of continuous light.

Plastid-expressed hCT-1 is bioactive

To test the biological activity of recombinant hCT-1 produced in chloroplasts, a human hepatocarcinoma cell line, HepG2, was used. In this cell line, the ability of rhCT-1 to induce phosphorylation of the signal transducer and activator of transcription-3 (STAT-3) was measured. Non-stimulated cells were used as a negative control. Phosphorylation of STAT-3 (P-STAT-3) was assessed by immunoblot analysis. The crude extracts from wild-type and transplastomic PpsbA-CT1 plants were added to the HepG2 cell culture medium. As a positive control, commercial hCT-1 was used. Wild-type plant extract showed no activity in this assay (Figure 6a, top), indicating that there is no endogenous plant compound that can phosphorylate the STAT-3 protein. In contrast, the PpsbA-CT1 plant extract induced such phosphorylation, although the extent of P-STAT-3 induction by transgenic plant extracts, harvested after 30 h of continuous light, was significantly lower than that expected from the recombinant protein concentration used in the assay (Figure 6a, top). In addition, the pattern shown by actin band intensities (Figure 6a, bottom) indicates that the observed differences in P-STAT-3 levels are not a result of loading variability. Densitometric analysis was performed to quantify the P-STAT-3 protein. Although rhCT-1 amounts increased by over two-fold under 32 h of continuous light (Figure 5a), the ability of rhCT-1 obtained from these plants to induce P-STAT-3 was significantly lower (~35%) than the ability of commercial hCT-1 (Figure 6b). However, only a slight decrease in rhCT-1 bioactivity was observed when leaves were harvested under dark conditions, or after 12 h of light (Figure 6b). One of the most deleterious effects of environmental stress (i.e. continuous light) on plants is 'photo-oxidative stress', which is primarily induced by the large absorption of excitation energy, leading to over-reduction of the electron transport chains. As a result, harmful reactive oxygen species (ROS) build up in tissues (Reddy and Raghavendra, 2006), therefore probably affecting rhCT-1 bioactivity. These oxidative conditions could also alter rhCT-1 folding and bioactivity. In this context, it is interesting to note that no experimental evidence exists on disulphide bridge formation in human CT-1 protein.
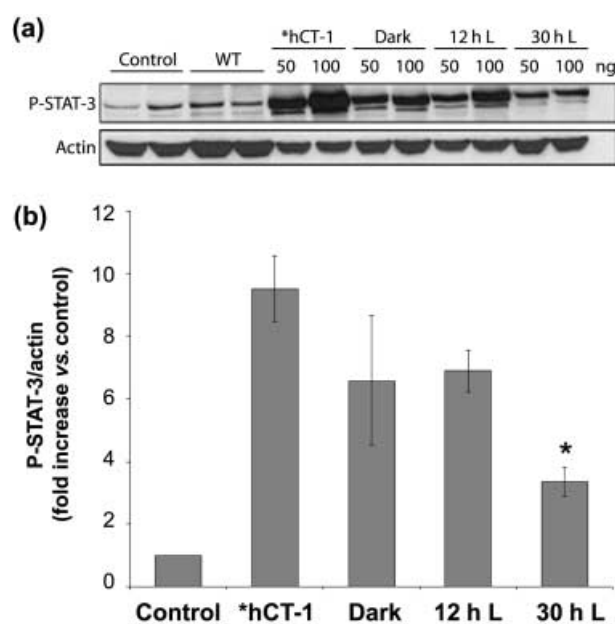

Figure 6 Bioactivity of recombinant human cardiotrophin-1 (rhCT-1) produced in tobacco chloroplasts. (a) Representative Western blot for signal transducer and activator of transcription-3 phosphorylation (P-STAT-3) (Tyr705) and actin (loading control) in HepG2 cell line stimulated with 50 and 100 ng per well of commercial hCT-1 (*hCT-1) or equivalent recombinant hCT-1 amounts from the crude extract of PpsbA-CT1 leaves harvested in darkness (Dark) of under 12 h (12 h L) and 30 h (30 h L) of light. Cells treated with wild-type crude extract (WT) and non-stimulated cells (Control) were used as negative controls. (b) Densitometric analysis of P-STAT-3 in stimulated HepG2 cell line extracts. The expression levels are presented as the fold increase vs. control (= 1) using the ratios between the densities of the P-STAT-3 bands and the corresponding actin bands. The data are presented as the mean ± standard error of two individual experiments. *P = 0.1 vs. commercial hCT-1.

In addition, published CT-1 sequences, such as those from rat and mouse, have only one cysteine, suggesting that the disulphide bridge linkage is not essential for activity. Perhaps the over-oxidizing conditions arising under 32 h of continuous light could lead to modifications of the recombinant protein structure and its bioactivity. Such a decrease in protein bioactivity has never been described previously in chloroplasts expressing therapeutic proteins under continuous light (Arlen *et al*., 2007; Ruhlman *et al*., 2007). To our knowledge, this is the first time that the bioactivity of a chloroplast-made therapeutic protein has been shown to be significantly affected by over-lighting, probably as a result of specific features of the CT-1 protein. Indeed, oxidative stress has been found to stimulate CT-1 release to the extracellular milieu from isolated hepatocytes (Iñiguez *et al*., 2006), whereas, under non-stress conditions, CT-1 is found inside the cell. Ateghang *et al*. (2006) also described the regulation of CT-1 expression in mouse embryonic stem cells by
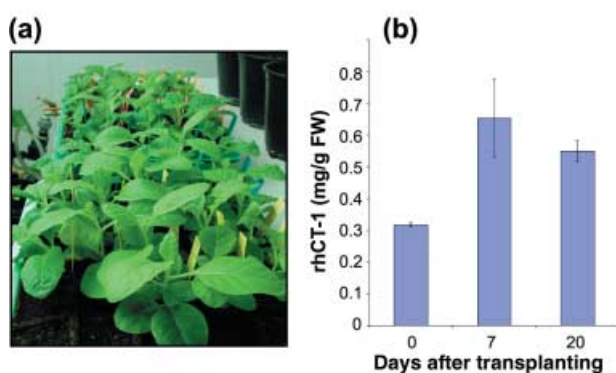
Figure 7 Production of recombinant human cardiotrophin-1 (rhCT-1) in seedlings. (a) Seedlings, germinated and grown *in vitro* for 4 weeks, were transferred to soil in jiffy pots until harvest. (b) Quantification of rhCT-1 expression by enzyme-linked immunosorbent assay (ELISA). Total soluble protein was extracted from seedling plants grown in a walk-in room and harvested at different days after transplantation (0, 7 and 20 days). Expression levels are indicated as milligrams of rhCT-1 per gram fresh weight (FW). The data are presented as the mean ± standard error of measurements on four individual seedling plants.

intracellular ROS. To assess these results, further studies will be specifically addressed in future research.

Scaling up the production of rhCT-1 in seedlings

To evaluate the feasibility of using these plants for commercial purposes, the production of rhCT-1 in seedlings grown in a walk-in room under a 16-h photoperiod was also examined (Figure 7a). The amount of rhCT-1 accumulated in PpsbA-CT1 plantlets harvested at different times after transplantation was quantified by ELISA (Figure 7b). Levels of rhCT-1 produced in 7- and 20-day-old plantlets (0.65 and 0.55 mg/g FW, respectively) were approximately two-fold higher than those produced in plantlets just after *in vitro* culture (0.32 mg/g FW).

A 'closed commercial production system' was commercialized in Japan in 2002 for the production of horticultural crop seedlings (Kozai, 2005). This system offers several advantages over seedling production in glasshouses. The most important is the higher productivity (~10-fold) per floor area per year, an effect that is mainly attributable to the high plant

density (up to 1500 seedlings/m^2) and shorter production period (20 days). Each unit of the commercial-type closed system has a floor area of 22 m^2, thus producing 33 000 seedlings in one batch; this amounts to 594 000 seedlings per year (one cycle lasts 20 days, permitting 18 batches/year) (Kozai, 2005). We were able to generate 4.2 mg of rhCT-1 from a single plantlet grown in a walk-in room when harvested 20 days after transplantation (Table 1). From the observed expression levels, it should be possible to obtain yields of up to 2.5 kg of rhCT-1 per unit of the commercial-type closed system and year, taking into account the high plant productivity described above. Furthermore, by increasing the harvest frequency to 7 days, despite decreasing yields per plantlet (1.9 mg/plantlet; Table 1), it should be possible to produce up to 3.2 kg of rhCT-1 per unit of the commercial-type closed system and year (52 batches/year). One of the potential therapeutic uses of CT-1 is as a hepatoprotective in liver transplantation surgery (Bustos *et al*., 2003) (procedures that are carried out at a rate of about 1000 per year in Spain, 4500 in the USA, 5000 in Europe and more than 15 000 per year all around the world). A dose of recombinant hCT-1 for this purpose could be about 14–28 mg for an average 70-kg individual. Based on a potential purification efficiency of 50%, more than 45 000 doses per year could be produced in a single commercial-type closed system, a yield that far exceeds the estimated worldwide needs of hCT-1 for liver transplantation surgery. However, this cytokine might also be applied as a tissue protective factor against ischaemia–reperfusion injury in the transplantation of other organs (heart, kidney, etc.), and to preserve the function of the remnant liver after surgery for primary and metastatic liver cancer (Bustos *et al*., 2003). New potential therapeutic uses continue to appear (e.g. Alzheimer's disease), raising interest in the development of efficient production systems.

The commercial potential of molecular farming of plant-made pharmaceuticals is regulated to ensure the protection of both the environment and consumers. The chloroplast transformation system provides for transgene containment from pollen transmission. However, two recent articles (Ruf *et al*., 2007; Svab and Maliga, 2007) have reported pollen transmission in the range 0.0087%–0.00024% in tobacco

Plantlet age (days after transplantation)	rhCT-1 in fresh plantlet (mg/g)	Average weight per plantlet (g)	Yield of rhCT-1 (mg/plantlet)
7	0.65 ± 0.12 (*n* = 4)	2.9 ± 0.36 (*n* = 10)	1.9
20	0.55 ± 0.03 (*n* = 4)	7.7 ± 0.64 (*n* = 10)	4.2

Table 1 Yield of recombinant human cardiotrophin-1 (rhCT-1) protein expressed in chloroplasts of transgenic tobacco plantlets grown in a walk-in room

Data are the mean ± standard error; number of replications given in parentheses.

plants. In our study, a confined method is proposed for the production of recombinant proteins in seedling plants in an environmentally friendly manner, thereby eliminating the potential hazard of pollen or seed spread.

In conclusion, a high-density seedling expression system is described to over-produce bioactive rhCT-1, as a His-tag fusion protein with enterokinase cleavage site, in tobacco chloroplasts. Although no attempt has yet been made to cleave the purification tag, the expression of this fusion protein will probably allow the production of tag-free pure fractions, which is essential for further clinical studies. The plastid transformation vector used in this work is equipped with *loxP* sequences, thus providing an efficient system to obtain transplastomic plants free of the *aadA* spectinomycin resistance gene.

Experimental procedures

Genetic engineering of chloroplast expression vectors

Tobacco chloroplast transformation vectors were based on pBluescript SK (Stratagene, La Jolla, CA, USA), with the elements necessary for plastid transformation inserted between the *Sac*I and *Kpn*I restriction sites. The *aadA* coding sequence fused to the *rrn* promoter (P*rrnaadA*) was obtained from plasmid pF*aadA*II (Eibl *et al.*, 1999). *rhct1* (bp 38–643 according to GENBANK accession number BC064416) was amplified by PCR from the IRAKp961M02139Q clone (RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH, Berlin, Germany). Other elements were amplified by PCR from *Nicotiana tabacum* plastid DNA (bp positions according to GENBANK accession number NC_001879): flanking regions, bp 138 544–140 315 and 140 316–141 475; *psbA* termination sequence (T*psbA*), bp 140–534; *rps16* termination sequence (T*rps16*), bp 4939–5087; *psbA* promoter and its 5′-UTR (P*psbA*), bp 1598–1785; 16S rRNA promoter (P*rrn*), bp 139 982–140 065; *psbA* promoter, bp 1683–1785. The complete *aadA* selection cassette was flanked by two *loxP* sites inserted by cloning primers. The His tag enterokinase sequence (5′-ATGGGTCACCATCACCATCACCATGACGATGACGATAAG-3′) and the leader sequence of phage T7 *G10*L (bp 22 904–22 966 according to GENBANK accession number NC_001604) were amplified by PCR-based gene assembly using overlapping primers. Finally, P*rrnG10*L and P*psbAG10*L were produced by overlap extension PCR. The complete sequences of the final vectors pL3-PpsbA-CT1, pL3-PrrnG10L-CT1 and pL3-PpsbAG10L-CT1 are available from GENBANK (accession numbers EU520589, EU520587 and EU520588, respectively). Construction details are available on request. Before proceeding with bombardment, the vectors were tested by Western blot analysis in *E. coli*.

Bombardment and regeneration of chloroplast transgenic plants

Gold microprojectiles coated with plasmid DNA (pL3-PpsbA-CT1, pL3-PrrnG10L-CT1 or pL3-PpsbAG10L-CT1) were bombarded into tobacco (*N. tabacum* var. Petit Havana) *in vitro*-grown leaves using the PDS1000/He (Bio-Rad, Hercules, CA, USA) biolistic device, as described previously (Daniell, 1997). After bombardment, the leaves were incubated in the dark for 2 days at 28 °C. The leaves were then cut into small pieces (~5 mm × 5 mm) and placed, adaxial side up, on selection medium (RMOP) in Magenta vessels (Sigma, St Louis, MO, USA) containing 500 mg/L spectinomycin dihydrochloride as selecting agent. The growth conditions of the culture chamber were 28 °C, 120 μmol/m²/s and a 16-h photoperiod. Spectinomycin-resistant shoots obtained after 6–7 weeks were cut into small pieces (~2 mm × 2 mm) and subjected to successive rounds of selection in the same selection medium. After three to four rounds, regenerated plants were transplanted and grown in a walk-in room under the following conditions: 28 °C, 250 μmol/m²/s, 70% relative humidity and 16-h photoperiod.

Southern and Northern blot analyses

Total plant DNA (10 μg) was digested with *Bgl*II, separated on a 0.8% (w/v) agarose gel and transferred to a nylon membrane. A 0.8-kb probe (P1), homologous to the flanking sequences, was obtained by PCR using tobacco DNA as template and the following primers: 5′-CCAAGGCATAACTTGTATCC-3′ and 5′-GAGTGTAC-CATGAGAGAAGC-3′. The pSK-PrrnG10L-CT1 vector, digested with *Sal*I and *Nco*I, generated a 0.7-kb probe (P2) homologous to the *rhct1* sequence. Probe labelling and hybridization were performed using the chemiluminescent AlkPhos direct labelling detection system (GE Healthcare, Buckinghamshire, UK). After Southern blot analysis confirmation, the plants were transferred to soil. Seeds from the T₀ generation were *in vitro* germinated on spectinomycin selection medium. The T₁ seedlings were isolated and cultured for 2 weeks in Magenta vessels. Finally, plants were transferred to pots. Plants of the T₀ and T₁ generations were analysed for homoplasmy.

Total RNA was extracted (Ultraspec RNA, Biotecx Laboratories, Houston, TX, USA) from the leaves of transformed and untransformed plants. RNA (5 μg) was separated on 1.5% agarose/formaldehyde gels and then transferred to a nylon membrane. P2 was used as the hCT-1-specific probe (described above). Constitutive 16S probe (150-bp) was generated by PCR using tobacco DNA as template and the following primers: 5′-CTTTTTAAGTCCGCCGTCAAA-3′ and 5′-TCTTTCCGATCTCTAC GCATTTC-3′. Labelling and hybridization were performed using the chemiluminescent detection system mentioned above.

Transcript quantification by real-time PCR

Transcript quantification was performed by real-time PCR. Four independent plants for the PpsbA-CT1 and PrrnG10L-CT1 constructs (three independent plants for PpsbAG10L-CT1) and three biological replicates per plant were analysed. Untransformed plants grown under the same conditions were used as controls. Total RNA was extracted (Ultraspec RNA, Biotecx Laboratories) and analysed by the SyBR Green protocol (Applied Biosystems, Foster City, CA, USA) at Scientific Park (Universidad Autonoma de Madrid, Madrid, Spain). The primers for *rhct1* detection were 5′-TCTCACTTCTTCCCCACTTGGA-3′ and 5′-CAAGGCTGTGTGTCTGACGG-3′. The *aadA* transcript was used as constitutive control of the plastid RNA loaded per reaction. The primers for *aadA* detection were 5′-ATCATTCCGTGGCGTTATCC-3′ and 5′-CCAAGGCAACGCTATGTTCTC-3′.

Western blot analysis

Transformed and untransformed leaves of plants grown in a walk-in room were ground in liquid nitrogen. TSP was extracted as described previously (Farran *et al.*, 2002). Leaf extracts were electrophoresed in a 13% polyacrylamide gel, and separated proteins were transferred to a nitrocellulose membrane for immunoblotting. Commercial hCT-1 protein (PeproTech, London, UK) was used as positive control. The primary antibody (monoclonal anti-hCT-1; R&D Systems, Minneapolis, MN, USA) was used at a 1 : 2000 dilution, and the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G; Pierce, Rockford, IL, USA) was used at a 1 : 5000 dilution. Detection was performed using the enhanced chemiluminescence (ECL) Western blotting system (GE Healthcare).

ELISA quantification of recombinant protein

Transformed and untransformed leaves (100 mg of each) from plants grown in a walk-in room were ground in liquid nitrogen, resuspended in 500 μL of extraction buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 0.1% Triton X-100 and cocktail of anti-protease) and incubated for 30 min on ice. The supernatant obtained after 10 min of centrifugation at 20 800 *g* contained the soluble fraction. The process was performed at 4 °C. Sandwich ELISA was used to determine the recombinant hCT-1 concentration in these samples. Capture antibody (monoclonal anti-hCT-1 antibody; R&D Systems), at 4 μg/mL of phosphate-buffered saline (PBS), was bound to a 96-well polyvinyl chloride microtitre plate (Costar Corning, Corning, NY, USA) overnight at room temperature. Background was blocked with 1% (w/v) bovine serum albumin (BSA) and sucrose in PBS for 2 h at room temperature. Plates were incubated with samples or standards diluted in buffer [20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.4, 150 mM NaCl, 0.1% BSA, 0.05% Tween-20] for 2 h at room temperature. The plates were then incubated with 200 ng/mL of a biotinylated anti-hCT-1 antibody (R&D Systems) in blocking solution (2 h at room temperature), followed by incubation with streptavidin–horseradish peroxidase conjugate (Amersham) for 1 h at room temperature. Washes were performed between each step with PBS containing Tween-20 (0.1%). Colour was developed by adding 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic) acid (ABTS; Roche, Mannheim, Germany), and the reactions were terminated after 1 h with 1% SDS. The absorbance at 405 nm was measured in a microplate reader (Multiskan Ex, Labsystems, Helsinki, Finland). A standard curve to calculate the amount of recombinant protein was made by plating a commercial hCT-1 (PeproTech) over a range of 40–125 ng per well. Transgenic leaf extracts were diluted to fit into the linear range of the hCT-1 standard. The total protein content of extracts was measured by the Bradford assay (Bio-Rad).

Bioactivity analysis of recombinant hCT-1

The human hepatoma cell line HepG2 was maintained in Dulbecco's modified Eagle's medium (Gibco, Paisley, UK) containing 10% fetal calf serum. For cell signalling experiments, cells were coated in six-well plates with 7.5×10^5 cells/well. After adhesion, cells were cultured in serum-free medium for 48 h, and then stimulated for 30 min with different amounts of commercial hCT-1 (PeproTech) or rhCT-1 from crude extracts of PpsbA-CT1s plants. Cells treated with wild-type plant extract and non-stimulated cells were used as negative controls. HepG2 cells were lysed for Western blot analysis in cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM KCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 10 mM sodium fluoride and cocktail of anti-proteases). Protein from lysates was heat denatured in double-strength SDS sample buffer containing dithiothreitol before resolution by SDS-PAGE. Western blot analysis was performed using specific antibodies for phosphorylated STAT-3 (Tyr705) (Cell Signalling, Beverly, MA, USA) and actin (Sigma). Densitometric analysis was performed by chemiluminescence detection using an ImageQuant ECL system and ImageQuant TL software (GE Healthcare). The expression levels are presented as the fold increase vs. control (= 1) using the ratios between the densities of the P-STAT-3 bands and the corresponding actin bands.

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Supplementary material

The following supplementary material is available for this article:

Figure S1 Homoplasmic plants expressing recombinant human cardiotrophin-1 (rhCT-1) under the control of the *psbA* promoter and the *G10* leader sequence.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1467-7652.2008.00334.x
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