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# Expression of Cholera Toxin B–Proinsulin Fusion Protein in Lettuce and Tobacco Chloroplasts – Oral Administration Protects Against Development of Insulitis in Non-Obese Diabetic Mice

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# Expression of Cholera Toxin B–Proinsulin Fusion Protein in Lettuce and Tobacco Chloroplasts – Oral Administration Protects Against Development of Insulitis in Non-Obese Diabetic Mice


## Abstract

Lettuce and tobacco chloroplast transgenic lines expressing the cholera toxin B subunit–human proinsulin (CTB-Pins) fusion protein were generated. CTB-Pins accumulated up to ~16% of total soluble protein (TSP) in tobacco and up to ~2.5% of TSP in lettuce. Eight milligrams of powdered tobacco leaf material expressing CTB-Pins or, as negative controls, CTB–green fluorescent protein (CTB-GFP) or interferon–GFP (IFN-GFP), or untransformed leaf, were administered orally, each week for 7 weeks, to 5-week-old female non-obese diabetic (NOD) mice. The pancreas of CTB-Pins-treated mice showed decreased infiltration of cells characteristic of lymphocytes (insulitis); insulin-producing β-cells in the pancreatic islets of CTB-Pins-treated mice were significantly preserved, with lower blood or urine glucose levels, by contrast with the few β-cells remaining in the pancreatic islets of the negative controls. Increased expression of immunosuppressive cytokines, such as interleukin-4 and interleukin-10 (IL-4 and IL-10), was observed in the pancreas of CTB-Pins-treated NOD mice. Serum levels of immunoglobulin G1 (IgG1), but not IgG2a, were elevated in CTB-Pins-treated mice. Taken together, T-helper 2 (Th2) lymphocyte-mediated oral tolerance is a likely mechanism for the prevention of pancreatic insulitis and the preservation of insulin-producing β-cells. This is the first report of expression of a therapeutic protein in transgenic chloroplasts of an edible crop. Transplastomic lettuce plants expressing CTB-Pins grew normally and transgenes were maternally inherited in T1 progeny. This opens up the possibility for the low-cost production and delivery of human therapeutic proteins, and a strategy for the treatment of various other autoimmune diseases.



## Keywords

autoimmune therapy, diabetes, edible crop, oral tolerance, plant-made pharmaceuticals


## Disciplines

Dentistry

## Comments

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# Field production and functional evaluation of chloroplast-derived interferon-α2b

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## Summary

Type I interferons (IFNs) inhibit viral replication and cell growth and enhance the immune response, and therefore have many clinical applications. IFN-α2b ranks third in world market use for a biopharmaceutical, behind only insulin and erythropoietin. The average annual cost of IFN-α2b for the treatment of hepatitis C infection is $26 000, and is therefore unavailable to the majority of patients in developing countries. Therefore, we expressed IFN-α2b in tobacco chloroplasts, and transgenic lines were grown in the field after obtaining United States Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS) approval. Stable, site-specific integration of transgenes into chloroplast genomes and homoplasmy through several generations were confirmed. IFN-α2b levels reached up to 20% of total soluble protein, or 3 mg per gram of leaf (fresh weight). Transgenic IFN-α2b had similar *in vitro* biological activity to commercially produced PEG-Intron™ when tested for its ability to protect cells against cytopathic viral replication in the vesicular stomatitis virus cytopathic effect (VSV CPE) assay and to inhibit early-stage human immunodeficiency virus (HIV) infection. The antitumour and immunomodulating properties of IFN-α2b were also seen *in vivo* . Chloroplast-derived IFN-α2b increased the expression of major histocompatibility complex class I (MHC I) on splenocytes and the total number of natural killer (NK) cells. Finally, IFN-α2b purified from chloroplast transgenic lines (cpIFN-α2b) protected mice from a highly metastatic tumour line. This demonstration of high levels of expression of IFN-α2b, transgene containment and biological activity akin to that of commercial preparations of IFN-α2b facilitated the first field production of a plant-derived human blood protein, a critical step towards human clinical trials and commercialization.

### Keywords

antitumour; antiviral; gene containment; molecular ‘pharming’; plant-made cytokine

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**Author contributions and site of performance**

PAA designed and contributed data for Figure 3a, Figure 4–Figure 6 and Table 2, interpreted the data and wrote the manuscript sections related to these investigations. RF created chloroplast transgenic lines (as a graduate student) and contributed to the data in Figure 1. SC contributed data to Figure 2 and Table 1 (as an undergraduate student). AC and AMC contributed the data in Figure 3b,c. KKO was responsible for the data on the field growth of transgenic plants. HD conceived and designed this study, secured NIH and USDA funding, interpreted the data, and wrote and revised several versions of the manuscript. All investigations reported here were performed at the University of Central Florida, except the field growth of transgenic lines that was conducted by investigators at Chlorogen, Inc., St. Louis, MO, USA.

## Introduction

The type I interferons (IFNs) are part of the body's first line of defence against viral attack, and also invasion by bacterial pathogens, parasites, tumour cells and allogeneic cells from grafts (De Maeyer and De Maeyer-Guignard, 1988). IFN-α and IFN-β display significant amino acid sequence homology (30%) and bind to the same receptor (Pestka *et al.*, 1987). Members of the IFN-α family were the first to be highly purified, sequenced, cloned and produced by recombinant DNA (Henco *et al.*, 1985). Although they are known for their inhibition of viral replication (Vilcek and Sen, 1996), IFN-α is also involved in regulating cytokine and cytokine receptor gene expression (Cousens *et al.*, 1997), mediating cellular proliferation and differentiation (Baron *et al.*, 1992), modifying immune cell distribution (Gresser *et al.*, 1981; Ishikawa and Biron, 1993) and activating natural killer (NK) cell cytotoxic activity (Trinchieri, 1989). Indeed, IFN-α is a potent inhibitor of cell growth (Pestka *et al.*, 1987). Perhaps most important, IFN-α may be a critical link between the innate and adaptive immune responses (Brassard *et al.*, 2002).

Recombinant IFN-α2b was first approved by the Food and Drug Administration (FDA) in 1986 for the treatment of hairy cell leukaemia. Today, it is routinely administered for the treatment of various cancers and viral diseases, but the cost of treatment is prohibitive in many developing countries (Stuart-Harris, 1997). The average cost of treatment using the marketed recombinant IFN-α2b is $26 000 for a 12-month course in the USA (Cowley, 2002).

The current therapeutic IFN-α2b is marketed under the names PEG-Intron™ and Intron®A and is made in the microbial system *Escherichia coli*. Many eukaryotic proteins cannot be expressed in prokaryotic hosts because the required formation of two disulphide bonds (Bodo and Maurer-Fogy, 1986) presents a major limitation, adding to the cost of production of the mature, biologically active IFN-α2b. As a result, IFN-α2b tends to aggregate to form inclusion bodies that need to be solubilized, renatured and refolded into an active protein (Swaminathan and Khanna, 1999). Other production platforms, such as silkworm using baculovirus (Maeda *et al.*, 1985) or phage vectors (Slocombe *et al.*, 1982), have been used to express IFN-α2b at low levels that are not competitive with the current system.

The plant nuclear genome has been used to express human therapeutic proteins for more than a decade (Horn *et al.*, 2004). The expression of IFN-α2b and IFN-α8 has been achieved by transforming potato nuclei (Ohya *et al.*, 2001). In tobacco, only 0.000017% fresh weight of transgenic leaves contained IFN-β (Edelbaum *et al.*, 1992). In addition, negligible amounts of IFN-α were produced in nuclear-transformed rice (Zhu *et al.*, 2004). The low and variable expression levels (caused by the position effect and gene silencing) using nuclear transformation are less than adequate to be commercially feasible, in addition to the problems of transgene containment that arise with field planting. Therefore, strategies are required to increase expression levels in plants and to facilitate transgene containment, allowing rapid and less expensive production of therapeutic proteins.

The chloroplast expression system is quite versatile in producing proteins as small as 20 amino acids, e.g. magainin (DeGray *et al.*, 2001), or as large as 135 kDa, e.g. Cry 1Ac (McBride *et al.*, 1995). Plastids can produce monomeric (Staub *et al.*, 2000; Fernandez-San Millan *et al.*, 2003) or multimeric proteins, including cholera toxin B (CTB) (Daniell *et al.*, 2001), CTB fusion proteins (Limaye *et al.*, 2006), and Guy's 13 (Daniell *et al.*, 2004a) and herpes simplex virus (Mayfield *et al.*, 2003) antibodies. Therapeutic proteins with 2–17 disulphide bonds, including human serum albumin (Fernandez-San Millan *et al.*, 2003) and somatotropin (Staub *et al.*, 2000), have already been produced in transgenic chloroplasts, with appropriate post-translational modifications, including lipid modifications, e.g. OspA lipoprotein (Glenz *et al.*, 2006). The chloroplast expression system has also been used to produce fully functional

vaccine antigens against bacterial pathogens, including cholera (Daniell *et al.*, 2001), anthrax (Watson *et al.*, 2004; Koya *et al.*, 2005) and tetanus (Tregoning *et al.*, 2003), viral pathogens, including canine parvovirus (Molina *et al.*, 2004) and rotavirus VP6 (Birch-Machin *et al.*, 2004), and protozoan pathogens, including amoeba (Chebolu and Daniell, 2006). The correct folding, disulphide bond formation and functionality of chloroplast-derived vaccine antigens and therapeutic proteins have been demonstrated by several assays, including macrophage lysis, GM1-ganglioside binding, systemic immune responses, protection against pathogen or toxin challenge and growth or inhibition of cell cultures (Daniell *et al.*, 2004a; Kamarajugadda and Daniell, 2006). Multiple genes have been engineered via the chloroplast genome in a single transformation event, facilitating the synthesis and assembly of multicomponent vaccines (Quesada-Vargas *et al.*, 2005). Therefore, any therapeutic protein, irrespective of size, can be made with desired post-translational modifications, with the notable exception of glycosylation (Daniell *et al.*, 2004a; 2005a). However, a few foreign proteins have been highly unstable within transgenic chloroplasts. For example, IFN-γ was degraded within chloroplasts (< 0.2% TSP), but expression of up to 7% TSP was achieved via fusion with β-glucuronidase (GUS) (Leelavathi and Reddy, 2003). Human insulin was similarly unstable in transgenic chloroplasts; fusion with CTB resulted in high-level expression (up to 16% TSP) and facilitated oral delivery studies to achieve protection against the development of insulitis in non-obese diabetic mice (Ruhlman *et al.*, 2007). Such N-terminal degradation is not unique to chloroplasts. All commercially produced insulin in bacteria or yeast is produced as a fusion protein; when expressed without fusion, insulin is rapidly degraded.

Plant chloroplasts are ideal bioreactors, and it has been shown that 1 acre of chloroplast transgenic plants can produce up to 360 million doses of clean, safe and fully functional anthrax vaccine antigen (Watson *et al.*, 2004; Koya *et al.*, 2005). This is a relatively rapid system for scale-up, because a single tobacco plant (a non-food, non-feed crop) produces up to one million seeds, which is adequate to plant more than 100 acres (8000 plants per acre and 40 metric tons of leaf biomass per acre). Transgenes integrated into the chloroplast genome are maternally inherited in most crops, ensuring that they are not spread via pollen, and thereby offering transgene containment (Daniell *et al.*, 1998; Daniell, 2002; Grevich and Daniell, 2005). Alternatively, cytoplasmic male sterility can be engineered via the chloroplast genome, thereby eliminating the production of viable pollen (Ruiz and Daniell, 2005). An additional advantage is that transformed seeds can be stored indefinitely and vaccine antigens can be produced on demand, eliminating the need to stockpile vaccine antigens under low temperature and with limited shelf life.

In this study, we investigate the expression of IFN-α2b in transgenic tobacco chloroplasts (cpIFN-α2b), and evaluate the functionality of purified IFN-α2b using commercial IFNs as comparative standards.

## Results and discussion

The 5.9-kb universal chloroplast vector (Daniell *et al.*, 2005b) used in this study contains several unique features that facilitate the integration of cloned DNA into the plastid genome. This process occurs exclusively through site-specific homologous recombination, thereby excluding the foreign vector DNA and enhancing transgene expression. The pLD-CtV vector incorporates the *trn*A and *trn*I genes (chloroplast transfer RNA transcripts coding for alanine and isoleucine, respectively) from the inverted repeat region of the tobacco chloroplast genome as flanking sequences for homologous recombination (Daniell *et al.*, 2005b). This vector was successfully used as the backbone for insertion of the expression cassette 5′UTR/HIS/THR/*IFNα2b* (Figure 1a).

### Selection and regeneration of transgenic lines

From seven bombarded Petit Havana leaves, 27 green shoots appeared after 4 weeks. From eight bombarded LAMD-609 leaves, 12 green shoots appeared within 7 weeks, indicating that the shoots from the low-nicotine tobacco LAMD-609 took longer to sprout and were less numerous. Untransformed cells appeared bleached on the antibiotic because they did not contain the *aad*A gene. For the second round of selection, the Petit Havana shoots were able to thrive on 500 μg/mL spectinomycin, whereas the LAMD-609 transformants only grew when the antibiotic concentration was decreased to 350 μg/mL spectinomycin.

### Confirmation of homoplasmy

For both tobacco varieties, Southern blots were performed to confirm integration of the *IFNα2b* cassette using appropriate DNA probes. A 0.81-kb DNA fragment containing the chloroplast *trn*I and *trn*A flanking sequences was used to probe total leaf DNA to determine homoplasmy or heteroplasmy after bombardment with pLD-RF-IFNα2b (Figure 1a). This determination was also used to estimate the chloroplast genome copy number. *Bam*HI-digested DNA from transformed plants produced a 9.9-kb fragment when probed (Figure 1b). Untransformed plant DNA from both tobacco varieties produced a single 7.9-kb fragment, indicating no integration of foreign DNA. All but one transgenic line exhibited only the 9.9-kb fragment (Figure 1b), indicating homoplasmy; plant #3c showed both the 7.9- and 9.9-kb fragments, indicating heteroplasmy. The attainment of homoplasmy in transformants provides a potential integrated transgene copy number of 10 000 copies per tobacco leaf cell (100 chloroplasts per plant cell × 100 genomes per chloroplast), and indicates that homoplasmy can be achieved in the $T_0$ generation. Transgene integration and maintenance of homoplasmy were demonstrated in subsequent generations ($T_1$, $T_2$, $T_3$, etc.; data not shown).

### Characterization of cpIFN-α2b

IFN-α2b is functional in its monomeric form, but has a tendency to aggregate (Wang, 1999). For LAMD-609, monomers of cpIFN-α2b protein were detected at approximately 21.5 kDa (Figure 1c), as a result of the presence of the polyhistidine tag and thrombin cleavage site, and smaller than the PEG-Intron™ standard (approximately 32 kDa). For Petit Havana, monomers of cpIFN-α2b protein were also detected at approximately 21.5 kDa (Figure 1d), slightly larger than the size of the Intron®A standard (19.2 kDa). In addition, dimers and multimers of cpIFN-α2b were observed in both LAMD-609 and Petit Havana.

The physical condition of the plants, its age and external factors, such as illumination and changes in temperature in the growth chamber and glasshouse, all contribute to the levels of protein expressed. Using enzyme-linked immunosorbent assay (ELISA), we examined the variation in cpIFN-α2b levels in young, mature and old leaves of the same transgenic plants of Petit Havana and LAMD, as well as under different durations of illumination, employing PEG-Intron™ and recombinant human IFN-α2b (rHu-IFN-α2b) as standards. In all transgenic lines grown in the glasshouse, higher levels of cpIFN-α2b expression were obtained when rHu-IFN-α2b was used as the standard (Figure 2). Old leaves showed the lowest levels of expression, corresponding to senescence inducing high proteolytic activity. Both young and mature leaves showed high levels of IFN-α2b expression, ranging from 1 to 3 mg/g fresh weight, or 8%–21% TSP, in $T_1$ LAMD transgenic lines (Figure 2a,b). In Petit Havana transgenic lines, IFN-α2b expression ranged from 0.2 to 1.2 mg/g fresh weight, or 2%–14% TSP (Figure 2c,d). The variation in expression levels may be the result of a decrease in cpIFN-α2b levels on continuous illumination in Petit Havana transgenic lines; in LAMD transgenic lines, such illumination increased IFN-α2b accumulation. This highlights the difference between Petit Havana and LAMD in the toleration of continuous light. The differences in protein levels correlate with differences in light intensity, as the same source of nutrition was provided to all transgenic lines. It is also known that 5′ untranslated regions of the *psbA* gene, located upstream of the

*IFNα2b* cassette, enhance the translation of the *psbA* gene in light (Eibl *et al.*, 1999). The dramatic difference in IFN-α2b levels in glasshouse-grown LAMD plants is most probably a result of light-regulated translational enhancement by the *psbA* regulatory sequences.

## Expression of cpIFN-α2b in field-grown biomass

In addition to the glasshouse, plants grown in the field were also examined. Approximately 2.5 months after transplantation in October, the biomass on stalks was cut 15 cm above ground with a forage harvester (Hege, Eging am See, Germany). From a single harvest, 107.7 kg of biomass was collected, shipped and stored in a commercial freezer (−20 °C) prior to analysis and purification. The plants had an average height of 43.2 cm and weighed 64 g. A single acre of tobacco biomass can be harvested four to six times within a growing season. As a result, there is the potential to generate greater than 1 metric ton of fresh biomass per acre under these conditions. As expected, Petit Havana plants had less height and weight per plant, and therefore less biomass per acre than commercial cultivars, which can generate up to 40 times more per acre (Cramer *et al.*, 1999). Based on 0.8 mg IFN-α2b per gram of leaf biomass, 107.7 kg of harvested biomass contained about 87.2 g of IFN-α2b. The limited yield was the result of a single harvest of biomass at a very early age because of premature flowering of Petit Havana in the field. Lower expression of IFN-α2b was anticipated in young leaves because the *psbA* regulatory sequence used to express IFN-α2b is developmentally regulated, and optimal levels of expression are achieved only in mature leaves of fully grown plants (Fernandez-San Millan *et al.*, 2003; Daniell *et al.*, 2004a; Watson *et al.*, 2004; Koya *et al.*, 2005; Limaye *et al.*, 2006).

We were able to generate 137.13 mg of IFN-α2b from 100 g of biomass at maturity in the glasshouse (Table 1). On the basis of observed expression levels, this should yield $5.5 \times 10^7$ mg of IFN-α2b in 1 acre of tobacco plants. A weekly dose of PEG-Intron™ (Schering Corporation, Kenilworth, NJ, USA), the *E. coli*-produced recombinant IFN-α2b treatment for hepatitis C, is 70 μg for an average 70-kg male. Based on a purification efficiency of 50%, approximately 400 million weekly doses for the treatment of hepatitis C can be produced in just 1 acre.

## Functional evaluation of cpIFN-α2b using vesicular stomatitis virus (VSV)

cpIFN-α2b activity was first evaluated by its antiviral properties. The activity of IFN-α2b is typically assayed by measuring its ability to inhibit the *in vitro* replication of VSV (Rubinstein *et al.*, 1981). Staining cells with crystal violet at the end of the assay enabled us to visualize the degree of cytopathicity under the microscope. Representative photographs are shown in Figure 3a. The mock controls (VSV alone and medium alone) were assigned protection values of 0% (complete cytopathicity) and 100% (complete protection), respectively. To rule out any toxic effects, IFN-α2b was tested in the absence of virus. As can be seen by the example of PEG-Intron™, cells grew normally in the presence of up to tenfold higher concentrations of IFN-α2b (Figure 3a). All experimental preparations of cpIFN-α2b protected cells, and did so in a dose-dependent manner. Purified cpIFN-α2b protected cells even when diluted 500-fold (data not shown). A crude extract of transgenic leaf material was still protective at the 1000-fold dilution (Figure 3a). Crude extracts of non-transgenic tobacco leaves were not protective, at any dilution, indicating that it is the presence of transgenic IFN-α2b in the plant that mediates the protection.

Crystal violet staining also enabled us to quantify the biological activity of the various IFN-α2b preparations tested. The activity is expressed in international units (IUs) and was calculated by comparing the levels of protection with controls. cpIFN-α2b from both laboratory and field sources was strongly protective (Table 2), as were the corresponding crude extracts. The weekly dose of PEG-Intron™ is also depicted in the table, to illustrate the levels of activity

achievable via the transgenic chloroplast system. As can be seen, 100 mg of transgenic leaf material expressing IFN-α2b has approximately the same biological activity as one weekly dose of PEG-Intron™.

## Functional evaluation of cpIFN-α2b using human immunodeficiency virus (HIV)

To further investigate the antiviral properties of cpIFN-α2b, we assessed the protection of cells from HIV-1 entry and integration. The targets for infection in this assay are TZM-BL cells, a derivative of HeLa cells that express CD4, CCR5 and CXCR4, rendering them excellent targets for infection by both tropic varieties of HIV. These cells also contain a luciferase expression cassette that is driven by the HIV long terminal repeat (LTR). Thus, when HIV productively infects TZM-BL, the high levels of Tat produced drive transcription by the endogenous LTR, resulting in quantifiable luciferase expression.

Two different HIV isolates were tested: the CCR5-tropic strain BaL and the CXCR4-tropic strain IIIB. PEG-Intron™ demonstrated approximately 80% inhibition of luciferase activity at both dilutions tested (Figure 3b,c). cpIFN-α2b from laboratory-derived plants showed nearly 100% protection from infection by both HIV strains at the 10-fold dilution; at the 100-fold dilution, protection from HIV BaL was nearly 60% (Figure 3b) and, from HIV IIIB, better than 80% (Figure 3c). cpIFN-α2b from field-derived plants also demonstrated strong protection: better than 65% for both dilutions and both strains tested.

Protection by transgenic plant crude extract was also noted. For HIV BaL, better than 50% protection was observed and, for HIV IIIB, better than 67% protection. Wild-type crude extract did not protect cells at all from infection by HIV BaL, but did provide minimal protection (< 20%) from HIV IIIB, indicating that it is the IFN-α2b expressed by transgenic leaves that provides the bulk of specific protection.

In summary, both forms of cpIFN-α2b tested (purified and crude extract, from both laboratory and field sources) were protective in *in vitro* antiviral assays. The successful performance of the transgenic plant crude extracts in the *in vitro* assays, with strong biological activity of cpIFN-α2b, demonstrates the advantage of the higher levels of expression observed in chloroplast transgenic lines. After performing initial *in vitro* studies using plant crude extracts, all subsequent investigations were conducted with purified cpIFN-α2b; the results observed were very similar to those of currently available commercial sources of IFN.

## *In vivo* studies

Amongst the myriad effects imparted by IFN-α2b, the up-regulation of major histocompatibility complex class I (MHC I) molecules (de Waard-Siebinga *et al.*, 1995) and the activation of NK cells (Gidlund *et al.*, 1978; Senik *et al.*, 1979) comprise a major part of its antiviral and antitumour properties. Our *in vivo* assays were designed to address whether our preparations of cpIFN-α2b were similarly capable of regulating the immune response.

**cpIFN-α2b up-regulates MHC I expression on splenocytes—**Blind screens of more than 100 sections of spleen tissue from each treatment condition were performed for the expression of cell surface markers. Initially, the expression of MHC I on splenocytes was analysed. Because all nucleated cells express MHC I antigens, we were interested in the relative differences between the treatment groups. The basal level of MHC I expression of mouse spleen tissue can be seen in the phosphate-buffered saline (PBS) treatment condition (Figure 4a). Treatment with cpIFN-α2b increased MHC I expression to levels similar to those achieved by PEG-Intron™ treatment. These results were confirmed by quantifying the immunofluorescence emission data. Spleen tissue isolated from PBS-treated mice demonstrated a mean fluorescence intensity (MFI) of MHC I expression of 74.7 units (Figure

4b). Mice treated with PEG-Intron™ had a significantly higher MFI (88.02 units; $P = 0.04$). Mice treated with cpIFN-α2b also had a significantly higher MFI (83.27 units; $P = 0.003$).

**cpIFN-α2b increases the number of NK cells *in vivo*—**Spleen tissue was also screened for the presence of NK cells. In PBS-treated mice, little staining of cells positive for the NK cell markers CD49b and NK1.1 was found (Figure 4a). By contrast, more positive NK cell staining was detected in mice treated with cpIFN-α2b. When the fluorescence emissions of the overlaid images were quantified, spleen tissue isolated from mice treated with PBS demonstrated a combined MFI ($MFI_{CD49b} \times MFI_{NK1.1}$) of 3797 units (Figure 4c). Mice treated with PEG-Intron™ showed a significantly higher combined MFI of 9366 units ($P = 1.6 \times 10^{-9}$). Mice treated with cpIFN-α2b also showed a significantly higher combined MFI of 8284 units ($P = 8.9 \times 10^{-15}$).

Finally, the splenocytes were analysed by flow cytometry. Representative plots are depicted in Figure 5a. Splenocytes isolated from mice treated with either PEG-Intron™ or cpIFN-α2b stained nearly 90% positive for MHC I expression (data not shown), and more than 6% were positive for the expression of the NK cell marker CD49b (Figure 5c). Mice treated with PBS showed significantly fewer splenocytes staining positive for CD49b ($P = 0.04$). By contrast, mice treated with cpIFN-α2b showed nearly 5.5% of splenocytes staining positive for CD49b, also a significant difference ($P = 1.3 \times 10^{-5}$). In addition, the MHC I-positive cell population from mice treated with cpIFN-α2b preparations showed a nearly 20% greater MFI than that of PBS-treated mice (Figure 5b), a significant difference (PEG-Intron™, $P = 0.01$; cpIFN-α2b, $P = 0.03$).

Taken together, these data lend support to the concept that cpIFN-α2b possesses the biological function of up-regulating MHC I expression and NK cell markers.

**cpIFN-α2b inhibits tumour metastasis *in vivo*—**IFN-α has well-characterized antitumour activity in malignancies (Gutterman, 1994; Belardelli *et al.*, 1998). The antitumour properties of cpIFN-α2b were assessed against B16-F10 cells, an aggressive metastatic melanoma cell line (Fidler *et al.*, 1976).

The degree of tumour metastasis could be determined empirically by macroscopic observation of the lungs after sacrifice (Figure 6a). Mice treated with PBS showed the highest degree of metastasis, indicated by near-complete coverage of the lung with tumour. By contrast, mice treated with either PEG-Intron™ or cpIFN-α2b exhibited quantifiably fewer black spots (Figure 6a).

These data were analysed further by calculating the colour intensity of the images, designed to help quantify the relative intensity of tumour (black) on a background of healthy lung tissue (red/pink). The calculations are summarized in Figure 6b, in which the mean colour intensity (MCI) of each group is plotted. As expected, mice treated with the positive control preparation (PEG-Intron™) had a mean MCI of 40.82 units, significantly higher than that of the animals treated with PBS (21.58 units; $P = 0.02$). cpIFN-α2b also significantly reduced the tumour metastasis to a mean MCI of 34.24 units ($P = 1.1 \times 10^{-7}$). These data suggest that the treatment of mice with cpIFN-α2b mediates antitumour activity and helps protect mice from the metastatic effects of the B16-F10 tumour line. Once again, as in previous assays, the performance of cpIFN-α2b is comparable with that of its commercial counterpart.

In conclusion, we have expressed IFN-α2b in tobacco chloroplasts, and transgenic lines were grown in the field after obtaining United States Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS) approval. Southern blots confirmed the stable, site-specific integration of transgenes into chloroplast genomes and homoplasmy in several

generations. Western blots detected monomeric and multimeric forms of cpIFN-α2b. ELISA showed up to 20% of TSP, or 3 mg IFN per gram of leaf (fresh weight). cpIFN-α2b possesses both *in vitro* and *in vivo* biological activity in both crude extract and purified forms. The induced up-regulation of MHC I molecules and activation of NK cells are consistent with the critical role played by IFN-α2b in early immune responses. MHC I is a necessary component of the antiviral response, increasing the presentation of foreign peptides to circulating immune cells primed for attack. NK cells survey the body for changes in MHC I expression, an indicator of abnormal situations, such as cancer. The priming of NK cells mediated by IFN-α2b helps to mobilize the immune system and facilitate the clearance of tumours. Our results provide a simple and cost-effective method for the production of functional IFN-α2b.

The commercial potential of molecular 'pharming' of plant-made pharmaceuticals has resulted in regulatory agencies formulating guidelines to protect the environment and consumers. Clearly, the rationale and risks of molecular pharming are very different from those of the first generation of crops genetically modified to enhance agronomic traits. In this study, we have used chloroplast genetic engineering to minimize the transmission of transgenes via pollen. We have also used a non-food/non-feed crop and expressed the therapeutic protein only in vegetative tissues. In addition, the transgenic plants were harvested before any reproductive structures appeared, thereby eliminating the spread of transgenes via pollen or seeds. This is the first example of a human blood protein produced in the field in transgenic plants. Large-scale production and purification in Good Manufacturing Practice (GMP) facilities should lead to human clinical trials and further advances in this field.

## Experimental procedures

### Construction of the pLD-RF-IFNα2b vector

The *IFNα2b* gene was purchased from the American Type Culture Collection (Gwynne *et al.*, 1993) (ATCC #53371; Manassas, VA, USA) in *E. coli* strain JM83 K-12. Sequencing results confirmed that the fragment in the pGL2BIFN vector was *IFNα2b*. Two overlapping primers were used at the 5′ end of the *IFNα2b* gene to include a thrombin cleavage site and a polyhistidine tag, with the reverse primer containing a *Not*I restriction site for further subcloning. After confirmation of the DNA sequence, the gel-eluted polymerase chain reaction (PCR) product was ligated into a Bluescript vector containing the 5′ untranslated region (UTR) of the *psb*A gene. This fragment (5′UTR/HIS/THR/*IFNα2b*) was inserted into the universal chloroplast vector pLD to create pLD-RF-IFNα2b. The recombinant DNA techniques were carried out as detailed previously (Sambrook *et al.*, 1989)

### Bombardment and selection of transgenic shoots

The Bio-Rad PDS-1000/He biolistic device (Bio-Rad, Hercules, CA, USA), a particle delivery system, was used to bombard tobacco leaves (Kumar and Daniell, 2004; Daniell *et al.*, 2005b). Two varieties of tobacco (*Nicotiana tabacum*) were generated for the bombardment: Petit Havana and LAMD-609 (low-nicotine hybrid produced by backcrossing a Maryland type variety, MD-609, with a low-nicotine-producing burley variety, LA Burley 21) (Collins *et al.*, 1974). After recovering in the dark for 48 h, leaves were cut under the hood into 5-$mm^2$ pieces and placed on regeneration medium of plants (RMOP) plates containing 500 μg/mL of spectinomycin for the first round of selection for transformants (Daniell, 1997; Daniell *et al.*, 2005b). Approximately 4 weeks later, the shoots were cut into 2-$mm^2$ pieces and transferred to fresh RMOP plates containing spectinomycin for a second round of selection. During this round of selection, the shoots that appeared were tested for cassette integration into the chloroplast genome by PCR analysis. Finally, after 4 weeks of secondary selection, the shoots were transferred to sterile jars containing fresh Murashige and Skoog medium without hormones (MS0) medium with 500 μg/mL spectinomycin (Kumar and Daniell, 2004; Daniell

*et al.*, 2005b). When the shoots grew to fill the jars, the transgenic lines were transferred to pots with soil containing no antibiotic. Potted plants were grown in a 16-h light/8-h dark photoperiod in the growth chamber at 26 °C or in the glasshouse or field.

### Southern blot analysis

Total plant DNA was extracted from transgenic $T_0$ plants and untransformed tobacco plants using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Total plant DNA was digested with *Hin*cII and probed by the flanking sequence probe, which was obtained from the pUC-Ct vector by digesting with *Bam*HI and *Bgl*II to yield a 0.81-kb fragment (Figure 1a). The probe was prepared by random primed $^{32}$P-labelling (Ready-To-Go DNA labelling beads; Amersham Biosciences, Pittsburgh, PA, USA). The probes were hybridized to the membrane using the Quick-hyb solution and protocol (Stratagene, La Jolla, CA, USA), as described previously (Kumar and Daniell, 2004; Daniell *et al.*, 2005b). The radiolabelled blots were exposed to X-ray films and then developed in the X-ray film processor.

### Immunoblot analysis

Plant extraction buffer (PEB) was made fresh on the same day as the Western analysis, and contained 100 mM NaCl, 10 mM ethylene-ediaminetetraacetic acid (EDTA) (pH 8), 200 mM Tris-HCl (pH 8), 0.05% Tween-20, 0.1% sodium dodecylsulphate (SDS), 14 mM β-mercaptoethanol (BME), 400 mM sucrose and 2 mM phenyl methyl sulphonyl fluoride (PMSF), which specifically inhibits serine proteases, such as chymotrypsin, trypsin and thrombin. From each transgenic Petit Havana and LAMD-609 line, leaf sections were cut and labelled as old (bottom), mature (middle) and young (top) leaves. Leaf material (100 mg) was ground in liquid nitrogen in cold, autoclaved mortars and pestles. PEB (200 μL) was added to each plant sample on ice and then vortexed for 10 s. A mouse antihuman IFN-α antibody (Abcam, Cambridge, MA, USA) was used for the immunoblot analysis of the extracted plant proteins.

### ELISA

Plant tissues were ground in liquid nitrogen using sterile mortar and pestles, and then placed on ice. Plant protein extraction buffer (500 μL of 15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, 3 mM $NaN_3$, 0.1% Tween-20, pH 9.6) was added to each sample and vortexed briefly. The samples were centrifuged at 4000 × ***g*** for 2 min and then placed back on ice. The supernatant was passed through a 0.22-μm filter and transferred to a fresh tube. Dilutions of crude extract ranging from 1 : 5 to 1 : 5000 were made in coating buffer (15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, 3 mM $NaN_3$, pH 9.6). PEG-Intron™ (a covalent conjugate of recombinant IFN-α2b with monomethoxy polyethylene glycol; Schering Corporation) was used as the standard and was also diluted in coating buffer. Aliquots of 100 μL of the diluted plant protein extract and standards were pipetted into a 96-well microtitre enzyme immunoassay (EIA) plate in duplicate. The plate was covered with parafilm and incubated at room temperature for 4 h. The wells were washed three times with 0.1% Tween-20 in phosphate-buffered saline (PBS-T), followed by three washes with deionized water. The plate was patted dry on paper towels, without letting the wells go completely dry. To detect the presence of IFN-α2b in each sample, 100 μL of mouse monoclonal antibody against human IFN (clone MMHA-2, PBL Biomedical, Piscataway, NJ, USA), diluted 1 : 2500 in PBS supplemented with 3% non-fat, powdered milk (Carnation) and 0.1% Tween-20 (P-T-M), was added to each well and incubated for 1 h at 37 °C. After incubation, the wells were washed as above, and 100 μL of the secondary antibody (goat anti-mouse immunoglobulin G) conjugated to horseradish peroxidase (American Qualex, San Clemente, CA, USA), diluted 1 : 5000 in P-T-M, was added to the wells and incubated for 1 h at 37 °C. After incubation, the wells were washed as above, and 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (American Qualex) was added to the wells. After allowing 5 min for colour change, 100 μL of 2 M sulphuric acid was added to stop the reaction.

The plate was immediately read on a microtitre plate reader (BioTek Instruments, Winooski, VT, USA) using a 450-nm filter.

### Estimation of TSP

The TSP of plant crude extract was determined by the Bradford assay. Bovine serum albumin (BSA; Sigma Chemical, St. Louis, MO, USA) was used as a standard in concentrations ranging from 0.05 to 0.5 mg/mL. Aliquots of 10 μL of diluted plant extract and each standard were added in duplicate to wells of a 96-well microtitre plate (Cellstar, Greiner, Nurtingen, Germany). Bradford reagent (Bio-Rad Protein Assay, Bio-Rad) was diluted 1 : 4 with distilled water, filtered, and 200 μL was added to each well and assayed as described previously (Kumar and Daniell, 2004; Daniell *et al.*, 2005b). The absorbance was read at 595 nm.

### Field production of biomass expressing IFN-α2b

IFN seeds in cultivar *Nicotiana tabacum* cv. 'Petit Havana' were initially propagated in the glasshouse using standard tobacco production practices for clipping, fertilizer and pesticide application suitable for field transplantation 30 days after seeding. The field was prepared prior to transplantation by the application of 138 kg/acre of ammonium nitrate using a Drop Spreader™ (Gandy Company, Owatonna, MN, USA), which was incorporated into the first 13 cm of soil using a Soil Finisher™ (John Deere, Moline, IL, USA). In August, seedlings were transplanted using a mechanical transplanter on non-raised beds in 40-cm rows with 30-cm plant spacings. The mean height of the transplanted seedlings was 15 cm, with an average of five leaves each, and planted 10 cm deep. Approximately 0.26 acre containing 7369 plants was seeded. At the time of transplantation, the field was prepared by applying insecticides [456 g/acre of Orthene (Monsanto, St. Louis, MO, USA) and 43 g/acre of Admire (Bayer CropScience, Research Triangle Park, NC, USA)] and herbicides [231 g/acre of Spartan (Chemical Products Technologies, Cartersville, GA, USA) and 922 g/acre of Command (FMC, Philadelphia, PA, USA)]. Crop maintenance required application of the fungicides Quadris (228 g/acre; Syngenta Crop Protection, Greensboro, NC, USA) and Acrobat MZ (48 g/acre; BASF, Research Triangle Park, NC, USA) every 20–30 days to control blue mould infestation, and a single application of Orthene (456 g/acre) to control aphid infestation.

### *In vitro* assays to study the functionality of IFN

**Cells—**Baby hamster kidney (BHK) and YAC-1 cells were obtained from ATCC. H9, PM1 and TZM-BL cells were obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (Germantown, MD, USA). BHK and TZM-BL cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). H9 cells were cultured in RPMI 1640 supplemented with 10% FBS and 100 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES). PM1 cells were maintained at a density of (4–8) × $10^5$ cells/mL in RPMI 1640 supplemented with 20% FBS and 100 mM HEPES. YAC-1 cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose and 1.5 g/L sodium bicarbonate.

**IFN preparation—**Crude extracts of transgenic and wild-type plants were generated using PEB as described above. Histidine-tagged transgenic cpIFN-α2b was purified under native conditions by passing crude extracts over HIS-Select spin columns (Sigma), according to the manufacturer's protocol. Flow-through, wash and eluate fractions were collected separately for analysis. PEG-Intron™ was used as the positive control for IFN-α2b activity. We routinely obtained greater than 85% purity of cpIFN-α2b (data not shown).

**VSV cytopathic effect (CPE) assay—**The Indiana strain of VSV was propagated in BHK cells (initial stocks were a kind gift from Dr Glen Barber, University of Miami, Miami, FL, USA). The virus was titrated by plaque formation on BHK cells. All viral dilutions were made in cell culture medium.

BHK cells were seeded in a 96-well plate 1 day prior to infection at a concentration of $1 \times 10^5$ cells/mL. Cells were treated for 6 h prior to infection with either crude extract or purified protein (25 μL), and then infected with 3000 plaque-forming units (PFUs) of VSV (25 μL); control wells were treated with either VSV alone or medium alone. The assay was stopped 24 h later. The medium from each well was aspirated, and the cells were fixed and stained by the addition of 100 μL of 0.5% (w/v in 70% methanol) crystal violet solution. After 1 min, the crystal violet solution was decanted and the wells were gently rinsed with water. After drying, the wells were examined under an inverted light microscope (Leica Microsystems, Wetzlar, Germany) and photographs were taken with a digital camera (Olympus America, Melville, NY, USA). The emission spectra of the crystal violet-stained wells were determined in a microplate reader at 570 nm.

**Titrating IFN—**The biological activity of the various preparations of IFN was calculated on the basis of that of PEG-Intron™ standards and expressed in international units (IUs). The mock controls (no IFN, no VSV) were assigned protection values of 0% and 100%, respectively. A formula was generated that correlated the optical density (OD) readings at 570 nm with the percentage protection of the mocks. Then, using the calculated biological activity of PEG-Intron™ ($7 \times 10^7$ IU/mg), a second formula correlating the percentage protection with the standardized IU of PEG-Intron™ was generated, and used to calculate the biological activity of all experimental samples.

**HIV-1 inhibition assay—**The HIV-1 laboratory strains BaL (R5) and IIIB (X4) were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. HIV-1 BaL was propagated in PM1 cells over 16 days. Supernatants containing virus were collected every other day, starting 5 days after infection, passed through a 0.45-μm pore size filter and stored in aliquots at −80 °C. HIV-1 IIIB was similarly propagated using H9 cells. Virus was quantified by ELISA for $p24^{gag}$ (Perkin-Elmer, Boston, MA, USA).

The protocol for this assay has been described elsewhere (Venkataraman *et al.*, 2005). TZM-BL cells, also known as JC53-BL cells (Wei *et al.*, 2002), which contain a luciferase expression cassette driven by the HIV LTR, were seeded in 96-well dishes (4000 cells/well). After 24 h, the cells were treated in triplicate with 50 μL of culture medium containing various preparations of IFN-α2b or vehicle control (PBS). Culture medium or virus diluted in culture medium (2 ng/mL p24 for BaL and 5 ng/mL p24 for IIIB) in 50 μL was immediately added to each well and allowed to incubate at 37 °C in 5% $CO_2$ for 24 h. Luciferase activity was subsequently measured with Bright-Glo reagents (Promega, Madison, WI, USA), according to the manufacturer's instructions, using an LMax luminometer (Molecular Devices, Sunnyvale, CA, USA). The cytotoxicity and the metabolic activity of the cells were verified by a tetrazolium-based (MTT) assay according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

### *In vivo* assays to study the functionality of IFN

**Mice—**Five-week-old C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Groups of mice were treated with PBS (three mice), PEG-Intron™ (five mice) or cpIFN-α2b (five mice) purified from transgenic tobacco leaves. Mice were treated with 20 000 IU of cpIFN-α2b diluted in 100 μL of PBS. The biological activity was based on the performance of purified protein in the VSV CPE assay. The doses were

administered daily for three consecutive days via intraperitoneal injection using a tuberculin syringe fitted with a 27-gauge needle. Mice were sacrificed 48 h after the final dose by carbon dioxide inhalation.

**Reagents—**All antibodies used for flow cytometry and primary antibodies for immunohistochemistry were obtained from BD Biosciences (San Jose, CA, USA); conjugated antibodies for immunohistochemistry were obtained from Molecular Probes (Eugene, OR, USA).

**Collection of tissue samples—**On sacrifice, mice were placed supine and the abdomen was opened along the ventral midline. A 23-gauge butterfly needle was placed in the right ventricle and sterile PBS was injected into the mouse for 5 min. One-half of the spleen was excised and immediately placed in cold culture medium (RPMI 1640 supplemented with 10% FBS). A solution of 4% paraformaldehyde (PFA) was then injected into the mouse via the right ventricle for 15 min. The remaining portion of the spleen was then excised and placed sequentially in 4% PFA, 10% sucrose, 20% sucrose and 30% sucrose, each for a period of 18–24 h. Samples were placed in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC, USA) and then flash frozen in 2-methylbutane (Sigma) over liquid nitrogen as fixed tissue specimens.

**Immunohistochemistry—**Sections of fixed frozen tissue (10 μm) were taken on a cryostat slicer (Microm HM505E; Mikron Instruments, San Markos, CA, USA) at −20 °C. The sections were mounted on Superfrost/Plus microscope slides (Fisher Scientific, Fair Lawn, NJ, USA). The slides were washed three times with PBS, and then blocked with PBS containing 10% BSA and 0.3% Triton X-100 for 1 h at room temperature. The sections were stained with one of the following primary antibodies (all antibodies were diluted 1 : 250 in PBS containing 1% BSA and 0.3% Triton X-100): mouse anti-H-$2K^b$ (clone AF6-88.5), rat anti-CD49b (clone DX5) or mouse anti-NK1.1 (clone PK136). The sections were incubated with primary antibody overnight at 4 °C. The slides were washed three times and then incubated with either of the following tagged secondary antibodies: rabbit anti-mouse/Alexa 488 or goat anti-rat Alexa 555. The sections were incubated with secondary antibody for 2 h at room temperature in the dark. The slides were washed three times; if double staining was required, the sequence of primary antibody staining, wash, secondary antibody staining and wash was repeated. At the end of the staining procedure, the slides were fixed by adding 50 μL of VectaShield Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). Coverslips were set and the slides were analysed on a Leica DM4000B fluorescent microscope (Leica Microsystems). For each filter cube used, samples were acquired at the same exposures to equilibrate light input. Then, using software-specific tools, the splenic tissue was isolated and the MFI of antibody staining was tabulated to account for both positively and negatively stained regions alike (Adobe Systems, San Jose, CA, USA).

***Ex vivo* preparation of splenocytes—**Spleen tissue in culture medium was dissociated using the plunger of a 10-mL syringe over a 70-μm cell strainer (BD Biosciences). The cells were washed once with PBS containing 2% FBS and pelleted by centrifugation at 150 × ***g*** for 10 min. The supernatant was aspirated and the cells were resuspended in culture medium at a concentration of $10^8$ cells/mL.

**Immunophenotyping—**Splenocytes were analysed for cell surface expression of the following markers: mouse anti-H-$2K^b$ (clone AF6-88.5) conjugated to fluorescein isothiocyanate (FITC), rat anti-CD49b (clone DX5) conjugated to phycoerythrin (PE) or mouse anti-NK1.1 (clone PK136) conjugated to allophycocyanin (APC). Samples were analysed on a FACSAria (BD Biosciences).

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## References

Baron, S.; Coppenhaver, DH.; Dianzani, F.; Fleischmann, WRJ.; Hughes, TKJ.; Klimpel, GR.; Niesel, DW.; Stanton, GJ.; Tyring, SKE. Interferon: Principles and Medical Applications. Galveston, TX: University of Texas Medical Branch; 1992.

Belardelli F, Ferrantini M, Santini SM, Baccarini S, Proietti E, Colombo MP, Sprent J, Tough DF. The induction of in vivo proliferation of long-lived CD44hi CD8+ T cells after the injection of tumor cells expressing IFN-alpha1 into syngeneic mice. Cancer Res 1998;58:5795–5802. [PubMed: 9865738]

Birch-Machin I, Newell CA, Hibberd JM, Gray JC. Accumulation of rotavirus VP6 protein in chloroplasts of transplastomic tobacco is limited by protein stability. Plant Biotechnol. J 2004;2:261–270. [PubMed: 17147617]

Bodo, G.; Maurer-Fogy, I. Characterization of different molecular species in affinity purified recombinant human interferon alpha 2. In: Dianzani, F.; Rossi, GB., editors. The Interferon System. New York: Raven Press; 1986. p. 23-27.

Brassard DL, Grace MJ, Bordens RW. Interferon-alpha as an immunotherapeutic protein. J. Leukocyte Biol 2002;71:565–581. [PubMed: 11927642]

Chebolu S, Daniell H. Stable expression of GAL/GALNAc lectin of *Entamoeba histolytica* in transgenic chloroplast and immunogenicity in mice towards vaccine development for amebiasis. Plant Biotechnol. J 2007;5:230–239. [PubMed: 17309678]

Collins GB, Legg PD, Kasperbauer MC. Tobacco hybrid LAMD-609. Crop Sci 1974;14:72–80.

Cousens LP, Orange JS, Su HC, Biron CA. Interferon-alpha/beta inhibition of interleukin 12 and interferon-gamma production in vitro and endogenously during viral infection. Proc. Natl. Acad. Sci. USA 1997;94:634–639. [PubMed: 9012836]

Cowley G. Hepatitis C. The insidious spread of a killer virus. Newsweek 2002;139:46–53. [PubMed: 11974394]

Cramer CL, Boothe JG, Oishi KK. Transgenic plants for therapeutic proteins: linking upstream and downstream strategies. Curr. Top. Microbiol. Immunol 1999;240:95–118. [PubMed: 10394717]

Daniell H. Transformation and foreign gene expression in plants mediated by microprojectile bombardment. Methods Mol. Biol 1997;62:463–489. [PubMed: 9108541]

Daniell H. Molecular strategies for gene containment in transgenic crops. Nat. Biotechnol 2002;20:581–586. [PubMed: 12042861]

Daniell H, Datta R, Varma S, Gray S, Lee SB. Containment of herbicide resistance through genetic engineering of the chloroplast genome. Nat. Biotechnol 1998;16:345–348. [PubMed: 9555724]

Daniell H, Lee S-B, Panchal T, Wiebe PO. Expression of the native cholera toxin B subunit gene and assembly as functional oligomers in transgenic tobacco chloroplasts. J. Mol. Biol 2001;311:1001. [PubMed: 11531335]

Daniell, H.; Carmona-Sanchez, O.; Burns, B. Chloroplast derived antibodies, biopharmaceuticals and edible vaccines. In: Fischer, R.; Schillberg, S., editors. Molecular Farming. Weinheim, Germany: Wiley-VCH-Verlag; 2004a. p. 113-133.

Daniell H, Kumar S, Dufourmantel N. Breakthrough in chloroplast genetic engineering of agronomically important crops. Trends Biotechnol 2005a;23:238–245. [PubMed: 15866001]

Daniell H, Ruiz ON, Dhingra A. Chloroplast genetic engineering to improve agronomic traits. Methods Mol. Biol 2005b;286:111–138. [PubMed: 15310917]

DeGray G, Rajasekaran K, Smith F, Sanford J, Daniell H. Expression of an antimicrobial peptide via the chloroplast genome to control phytopathogenic bacteria and fungi. Plant Physiol 2001;127:852–862. [PubMed: 11706168]

De Maeyer, E.; De Maeyer-Guignard, J. Interferons and Other Regulatory Cytokines. New York: John Wiley & Sons; 1988.

Edelbaum O, Stein D, Holland N, Gafni Y, Livneh O, Novick D, Rubinstein M, Sela I. Expression of active human interferon-beta in transgenic plants. J. Interferon. Res 1992;12:449–453. [PubMed: 1337755]

Eibl C, Zou Z, Beck A, Kim M, Mullet J, Koop HU. In vivo analysis of plastid psbA, rbcL and rpl32 UTR elements by chloroplast transformation: tobacco plastid gene expression is controlled by modulation of transcript levels and translation efficiency. Plant J 1999;19:333–345. [PubMed: 10476080]

Fernandez-San Millan A, Mingo-Castel A, Miller M, Daniell H. A chloroplast transgenic approach to hyper-express and purify human serum albumin, a protein highly susceptible to proteolytic degradation. Plant Biotechnol. J 2003;1:71–79. [PubMed: 17147744]

Fidler IJ, Gersten DM, Budmen MB. Characterization in vivo and in vitro of tumor cells selected for resistance to syngeneic lymphocyte-mediated cytotoxicity. Cancer Res 1976;36:3160–3165. [PubMed: 975082]

Gidlund M, Orn A, Wigzell H, Senik A, Gresser I. Enhanced NK cell activity in mice injected with interferon and interferon inducers. Nature 1978;273:759–761. [PubMed: 566386]

Glenz K, Bouchon B, Stehle T, Wallich R, Simon MM, Warzecha H. Production of a recombinant bacterial lipoprotein in higher plant chloroplasts. Nat. Biotechnol 2006;24:76–77. [PubMed: 16327810]

Gresser I, Guy-Grand D, Maury C, Maunoury MT. Interferon induces peripheral lymphadenopathy in mice. J. Immunol 1981;127:1569–1575. [PubMed: 7276571]

Grevich JJ, Daniell H. Chloroplast genetic engineering: recent advances and future perspectives. Crit. Rev. Plant Sci 2005;24:83–107.

Gutterman JU. Cytokine therapeutics: lessons from interferon alpha. Proc. Natl. Acad. Sci. USA 1994;91:1198–1205. [PubMed: 8108387]

Gwynne, DI.; Buxton, FP.; Pickett, MH.; Davies, RW.; Scazzocchio, C. Vectors in Use in Filamentous Fungi. Delft, NL: Royal Gist-Brocades N.V.; 1993.

Henco K, Brosius J, Fujisawa A, Fujisawa JI, Haynes JR, Hochstadt J, Kovacic T, Pasek M, Schambock A, Schmid J, Todokoro K, Wälchli M, Nagata S, Weissmann C. Structural relationship of human interferon alpha genes and pseudogenes. J. Mol. Biol 1985;185:227–260. [PubMed: 4057246]

Horn ME, Woodard SL, Howard JA. Plant molecular farming: systems and products. Plant Cell Rep 2004;22:711–720. [PubMed: 14997337]

Ishikawa R, Biron CA. IFN induction and associated changes in splenic leukocyte distribution. J. Immunol 1993;150:3713–3727. [PubMed: 7682583]

Kamarajugadda S, Daniell H. Chloroplast-derived anthrax and other vaccine antigens: their immunogenic and immunoprotective properties. Expert Rev. Vaccines 2006;5:839–849. [PubMed: 17184221]

Koya V, Moayeri M, Leppla SH, Daniell H. Plant-based vaccine: mice immunized with chloroplast-derived anthrax protective antigen survive anthrax lethal toxin challenge. Infect. Immun 2005;73:8266–8274. [PubMed: 16299323]

Kumar S, Daniell H. Engineering the chloroplast genome for hyperexpression of human therapeutic proteins and vaccine antigens. Methods Mol. Biol 2004;267:365–383. [PubMed: 15269437]

Leelavathi S, Reddy VS. Chloroplast expression of His-tagged GUS-fusions: a general strategy to overproduce and purify foreign proteins using transplastomic plants as bioreactors. Mol. Breed 2003;11:49.

Limaye A, Koya V, Samsam M, Daniell H. Receptor-mediated oral delivery of a bioencapsulated green fluorescent protein expressed in transgenic chloroplasts into the mouse circulatory system. Faseb J 2006;20:959–961. [PubMed: 16603603]

Maeda S, Kawai T, Obinata M, Fujiwara H, Horiuchi T, Saeki Y, Sato Y, Furusawa M. Production of human alpha-interferon in silkworm using a baculovirus vector. Nature 1985;315:592–594. [PubMed: 2989694]

Mayfield SP, Franklin SE, Lerner RA. Expression and assembly of a fully active antibody in algae. Proc. Natl. Acad. Sci. USA 2003;100:438–442. [PubMed: 12522151]

McBride KE, Svab Z, Schaaf DJ, Hogan PS, Stalker DM, Maliga P. Amplification of a chimeric Bacillus gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. Biotechnology (NY) 1995;13:362–365.

Molina A, Hervas-Stubbs S, Daniell H, Mingo-Castel AM, Veramendi J. High-yield expression of a viral peptide animal vaccine in transgenic tobacco chloroplasts. Plant Biotechnol. J 2004;2:141–153. [PubMed: 17147606]

Ohya K, Matsumura T, Ohashi K, Onuma M, Sugimoto C. Expression of two subtypes of human IFN-alpha in transgenic potato plants. J. Interf. Cytok. Res 2001;21:595–602.

Pestka S, Langer JA, Zoon KC, Samuel CE. Interferons and their actions. Annu. Rev. Biochem 1987;56:727–777. [PubMed: 2441659]

Quesada-Vargas T, Ruiz ON, Daniell H. Characterization of heterologous multigene operons in transgenic chloroplasts: transcription, processing, and translation. Plant Physiol 2005;138:1746–1762. [PubMed: 15980187]

Rubinstein S, Familletti PC, Pestka S. Convenient assay for interferons. J. Virol 1981;37:755–758. [PubMed: 6163873]

Ruhlman T, Ahangari R, Devine AL, Samsam M, Daniell H. Expression of cholera toxin B-proinsulin fusion protein in lettuce and tobacco chloroplasts – oral administration protects against development of insulitis in non-obese diabetic mice. Plant Biotechnol. J. 2007(OnlineEarly Articles)

Ruiz ON, Daniell H. Engineering cytoplasmic male sterility via the chloroplast genome by expression of {beta}-ketothiolase. Plant Physiol 2005;138:1232–1246. [PubMed: 16009998]

Sambrook, J.; Fritsch, EF.; Maniatis, T. Molecular Cloning – A Laboratory Manual. 2nd edn. New York: Cold Spring Harbor Laboratory Press; 1989.

Senik A, Gresser I, Maury C, Gidlund M, Orn A, Wigzell H. Enhancement by interferon of natural killer cell activity in mice. Cell Immunol 1979;44:186–200. [PubMed: 313257]

Slocombe P, Easton A, Boseley P, Burke DC. High-level expression of an interferon alpha 2 gene cloned in phage M13mp7 and subsequent purification with a monoclonal antibody. Proc. Natl. Acad. Sci. USA 1982;79:5455–5459. [PubMed: 6752948]

Staub JM, Garcia B, Graves J, Hajdukiewicz PT, Hunter P, Nehra N, Paradkar V, Schlittler M, Carroll JA, Spatola L, Ward D, Ye G, Russell DA. High-yield production of a human therapeutic protein in tobacco chloroplasts. Nat. Biotechnol 2000;18:333–338. [PubMed: 10700152]

Stuart-Harris, R.; Penny, RD., editors. Clinical Applications of the Interferons. London: Chapman & Hall Medical; 1997.

Swaminathan S, Khanna N. Affinity purification of recombinant interferon-alpha on a mimetic ligand adsorbent. Protein Expr. Purif 1999;15:236–242. [PubMed: 10049681]

Tregoning JS, Nixon P, Kuroda H, Svab Z, Clare S, Bowe F, Fairweather N, Ytterberg J, van Wijk KJ, Dougan G, Maliga P. Expression of tetanus toxin Fragment C in tobacco chloroplasts. Nucleic Acids Res 2003;31:1174–1179. [PubMed: 12582236]

Trinchieri G. Biology of natural killer cells. Adv. Immunol 1989;47:187–376. [PubMed: 2683611]

Venkataraman N, Cole AL, Svoboda P, Pohl J, Cole AM. Cationic polypeptides are required for anti-HIV-1 activity of human vaginal fluid. J. Immunol 2005;175:7560–7567. [PubMed: 16301665]

Vilcek, J.; Sen, GC. Interferons and other cytokines. In: Fields, BN.; Knipe, DM.; Howley, PM., editors. Fundamental Virology. New York: Lippincott-Raven Publishers; 1996. p. 341-365.

de Waard-Siebinga I, Creyghton WM, Kool J, Jager MJ. Effects of interferon alfa and gamma on human uveal melanoma cells in vitro. Br. J. Ophthalmol 1995;79:847–855. [PubMed: 7488605]

Wang W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. Int. J. Pharm 1999;185:129–188. [PubMed: 10460913]

Watson J, Koya V, Leppla SH, Daniell H. Expression of Bacillus anthracis protective antigen in transgenic chloroplasts of tobacco, a non-food/feed crop. Vaccine 2004;22:4374–4384. [PubMed: 15474731]

Wei X, Decker JM, Liu H, Zhang Z, Arani RB, Kilby JM, Saag MS, Wu X, Shaw GM, Kappes JC. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. Antimicrob. Agents Chemother 2002;46:1896–1905. [PubMed: 12019106]

Zhu Z, Hughes KW, Huang L, Sun B, Liu C, Li Y. Expression of human alpha-interferon cDNA in transgenic rice plants. Plant Cell Tiss. Org 2004;36:197–204.

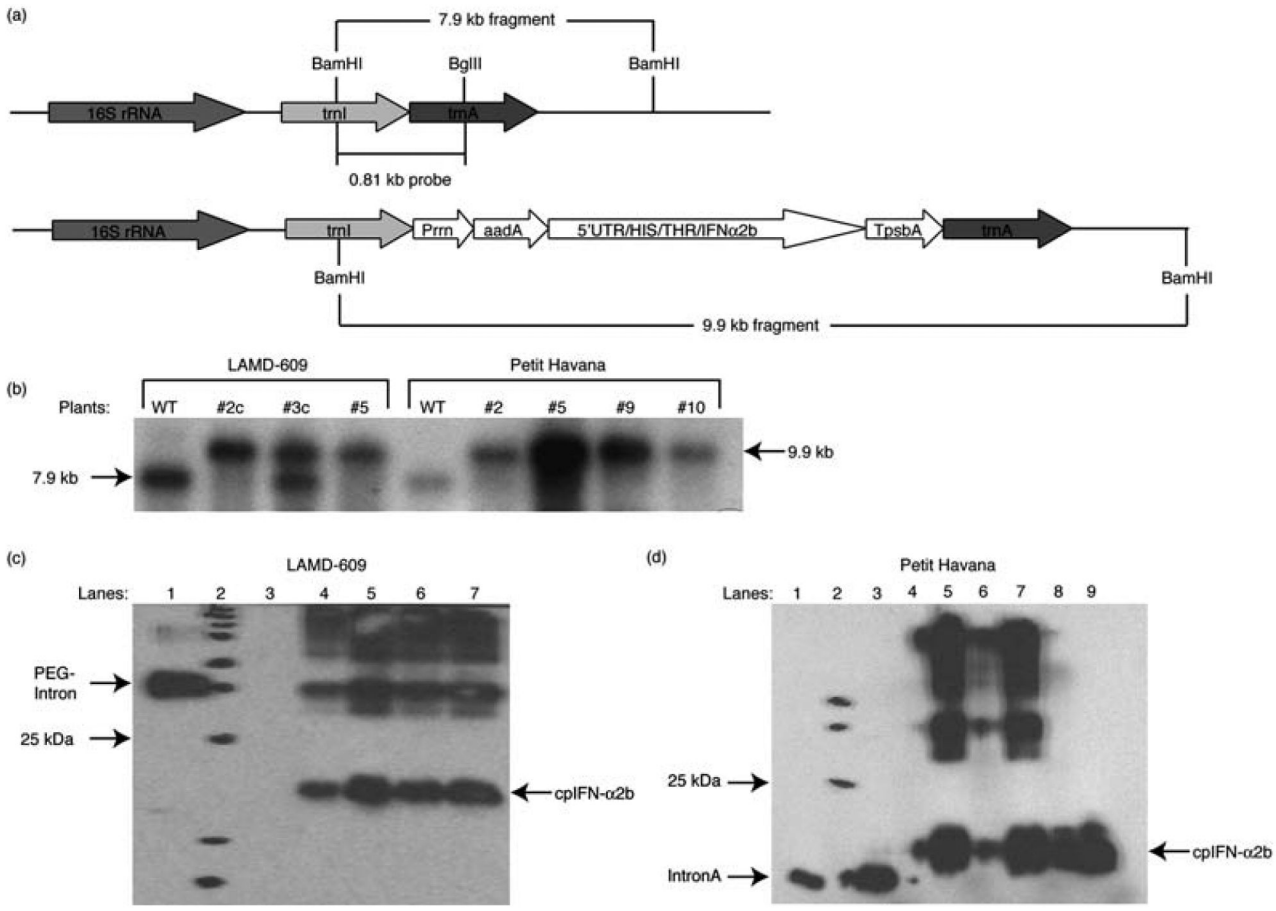


**Figure 1.**

Confirmation of chloroplast integration and determination of homoplasmy/heteroplasmy in the $T_0$ generation of both varieties. (a) The 0.81-kb DNA probe containing chloroplast flanking sequences. (b) DNA fragments of 7.9 kb indicate untransformed chloroplasts and DNA fragments of 9.9 kb are observed when the chloroplast genome contains the integrated transgenes. Lanes 1, 5, untransformed wild-type; lanes 2–4, transgenic LAMD-609 lines; lanes 6–9, transgenic Petit Havana lines. (c) Western blot of LAMD-609 transgenic plants expressing chloroplast-derived interferon-α2b (cpIFN-α2b). Low-nicotine tissue extract separated by 15% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), with cpIFN-α2b detected by mouse monoclonal antibody against human IFN-α. Lane 1, 80 ng PEG-Intron™ standard; lane 2, protein marker; lane 3, wild-type (untransformed) LAMD-609; lanes 4–7, transgenic LAMD-609 lines expressing monomers and multimers of cpIFN-α2b. (d) Western blot of Petit Havana transgenic plants expressing cpIFN-α2b. Petit Havana leaf extract separated by 15% SDS-PAGE, with cpIFN-α2b detected by mouse monoclonal antibody against human IFN-α. Lane 1, 38 ng of Intron®A; lane 2, protein marker; lane 3, 190 ng of Intron®A; lane 4, wild-type (untransformed) Petit Havana; lanes 5–7, transgenic Petit Havana lines expressing monomers and multimers of cpIFN-α2b; lanes 8, 9, *Escherichia coli*-transformed with IFN-α2b.

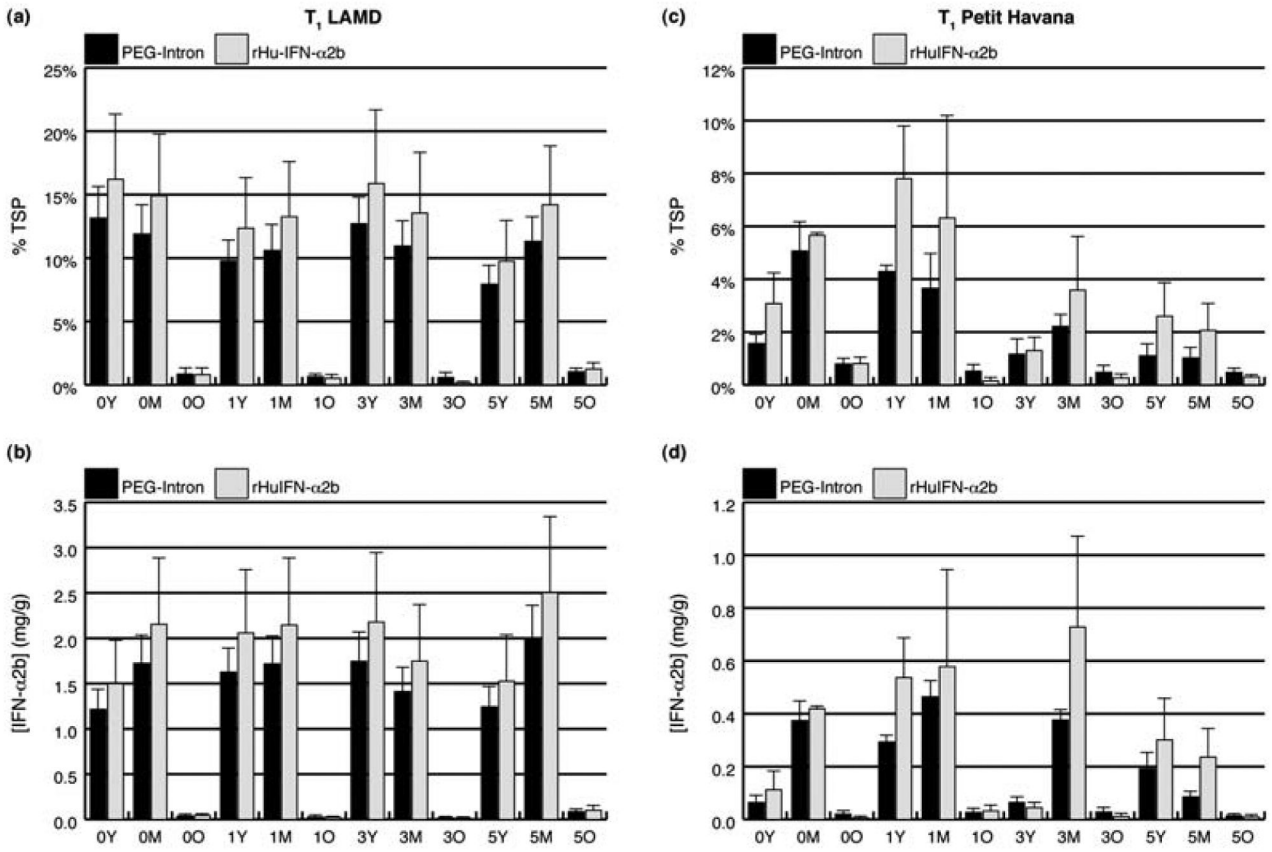


**Figure 2.**

Quantitation of interferon-α2b purified from chloroplast transgenic lines (cpIFN-α2b). Crude extracts of transgenic leaf material were analysed for IFN-α2b expression by indirect enzyme-linked immunosorbent assay (ELISA). Quantitation of transgenic IFN-α2b was performed on the $T_1$ generations of LAMD (a, b) and Petit Havana (c, d) plants. (a, c) Quantitation of cpIFN-α2b as a percentage of total soluble protein (TSP). (c, d) Quantitation of cpIFN-α2b as milligrams of transgenic protein per gram fresh leaf weight. Two different standards were used in the ELISA: recombinant IFN-α2b with a covalent conjugate of monomethoxy polyethylene glycol (PEG-Intron™) and recombinant human IFN-α2b (rHuIFN-α2b). Y, young leaves (top few in the plant); M, mature leaves (fully developed); O, old leaves (bottom senescent leaves with decreased pigments). Duration of illumination: 0, 1, 3 and 5 days of continuous illumination. Error bars represent the standard error of the mean.

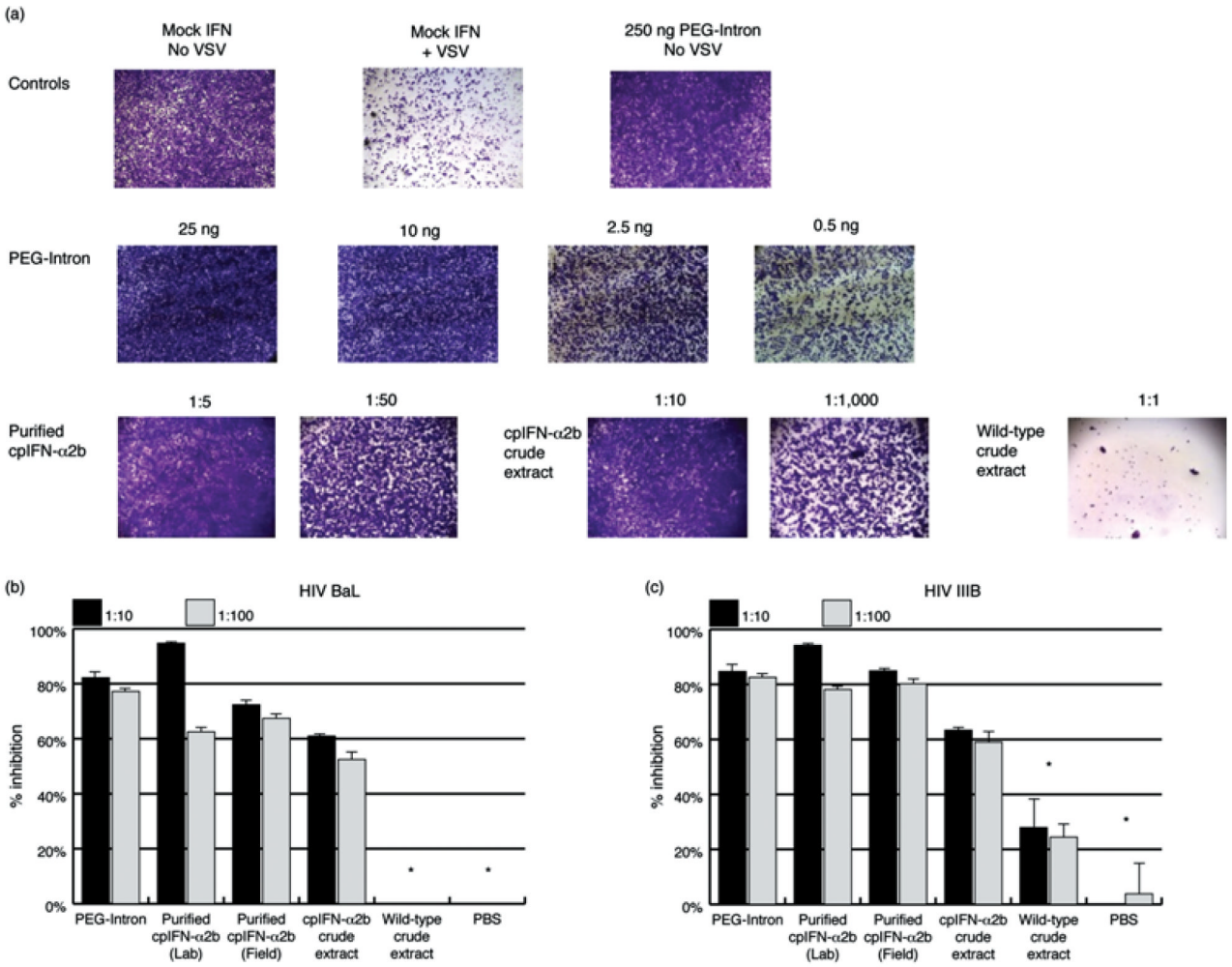


**Figure 3.**

Transgenic tobacco plants produce interferon-α2b (IFN-α2b) with biological activity *in vitro*. (a) Transgenic IFN-α2b inhibits vesicular stomatitis virus (VSV)-induced cytopathicity. Baby hamster kidney (BHK) cells were pretreated with the indicated samples for 24 h prior to VSV exposure. At the conclusion of the assay, the cells were washed and stained with crystal violet for microscopic examination. (b, c) Transgenic IFN-α2b inhibits human immunodeficiency virus (HIV) infection, as determined by a decrease in luciferase expression. TZM-BL cells were pretreated for 24 h with the indicated reagents, and then exposed to two different HIV isolates: the CCR5-tropic strain BaL (b) and the CXCR4-tropic strain IIIB (c). All treatments were performed in triplicate. Error bars represent the standard error of the mean. Asterisks indicate $P < 0.05$.

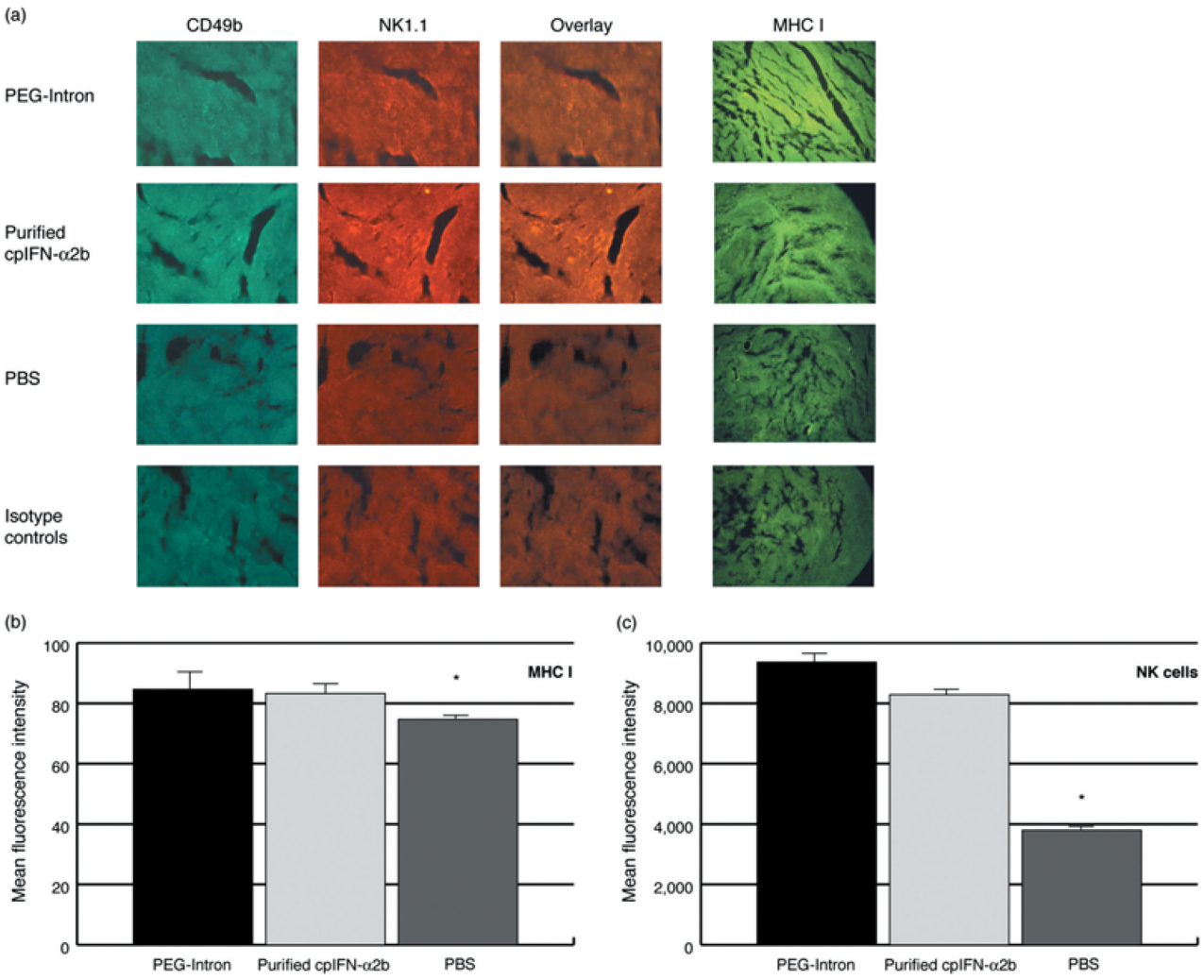


**Figure 4.**

Immunohistochemical detection of the interferon response in mouse spleens. (a) Representative photographs of mouse spleen tissue. The tissues were fixed and 10-μm sections were stained for either the natural killer (NK) cell markers CD49b and NK1.1 or major histocompatibility complex class I (MHC I). For NK cell marker staining, the channels were read individually and also overlaid. It should be noted that, because of the natural expression profile of MHC I, all cells are presumed to be positive. Therefore, these data are designed to evaluate relative differences between the conditions. Isotype controls depict the background fluorescence of the native tissue. Photographs were taken with a 20× objective and are representative of more than 100 sections of each treatment condition. (b, c) Analysis of the mean fluorescence intensity (MFI) of splenocytes stained for MHC I-positive cells (b) and NK cells (c). All tissue samples were acquired with equivalent MFI. Error bars in (b) and (c) represent the standard error of the mean. Asterisks indicate statistical comparisons between PEG-Intron™ and phosphate-buffered saline (PBS), and interferon-α2b purified from chloroplast transgenic lines (cpIFN-α2b) and PBS, with significant *P* values of less than 0.05.

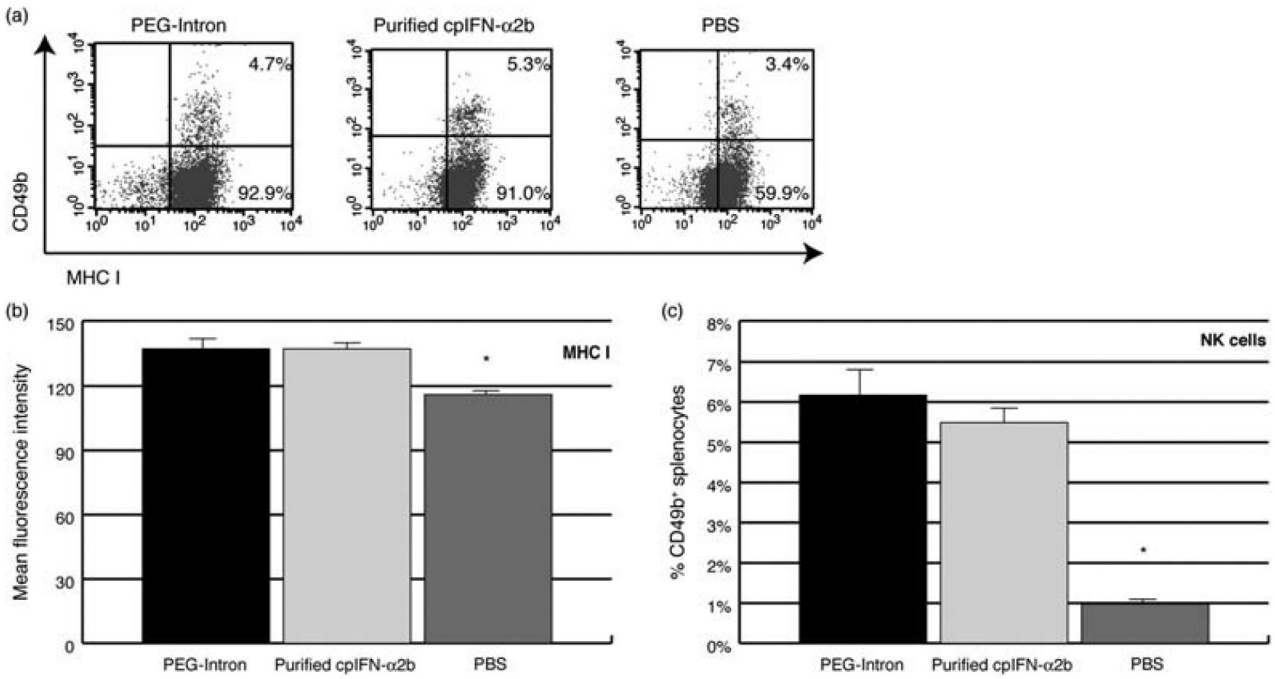


**Figure 5.**

Phenotypic analysis of splenocytes. Total splenocytes isolated from the various groups of mice in this study were analysed by flow cytometry for surface marker expression. (a) Representative flow cytometry plots showing splenocyte expression of major histocompatibility complex class I (MHC I) and CD49b. Quadrant positions are based on isotype controls. (b) Comparison of the mean fluorescence intensity (MFI) of MHC I across the various treatment groups. All cells comprising the MHC I-positive population (i.e. both CD49b-positive and CD49b-negative populations) are represented. (c) Comparison of the expression of natural killer (NK) cell markers (CD49b and NK1.1) across the various treatment groups. The data in (b) and (c) represent the means of each treatment group. Error bars represent the standard error of the mean. Asterisks indicate statistical comparisons between PEG-Intron™ and phosphate-buffered saline (PBS), and interferon-α2b purified from chloroplast transgenic lines (cpIFN-α2b) and PBS, with significant *P* values of less than 0.05.

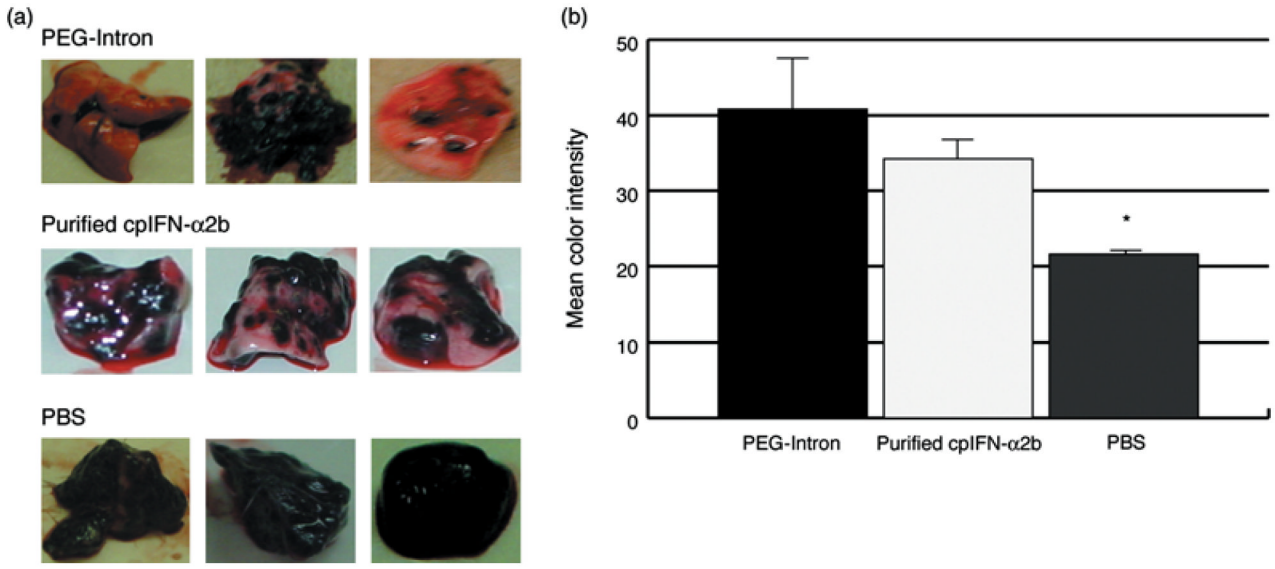


**Figure 6.**

Transgenic tobacco plants produce interferon-α2b (IFN-α2b) with biological activity *in vivo*. (a) Photographs of lungs of mice injected with the metastatic tumour B16-F10. Mice were treated via intraperitoneal injection, as indicated, for three consecutive days. (b) Mean colour intensity of tumour metastases. Photographs of lung samples were analysed for colour intensity. All samples were photographed with equal exposure settings. A calculation of total colour was then achieved by selecting only those pixels that corresponded to the lung tissue. The mean colour intensity was calculated for all samples of a particular group. The lower the intensity, the greater amount of black (tumour) in the image. Error bars represent the standard error of the mean. Asterisks indicate statistical comparisons between PEG-Intron™ and phosphate-buffered saline (PBS), and purified cpIFN-α2b and PBS, with significant *P* values of less than 0.05.

**Table 1**

Yield of interferon-α2b purified from chloroplast transgenic lines (cpIFN-α2b) in LAMD transgenic lines grown in the glasshouse

| Leaf age | Leaves per plant | IFN-α2b per leaf (mg) | Average weight per leaf (g) | IFN-α2b in fresh leaf (mg/g) | IFN-α2b per age group (mg) (no. leaves × [IFN-α2b] per leaf) |
|---|---|---|---|---|---|
| Young | 3.20 | 3.50 | 2.50 | 1.40 | 11.20 |
| Mature | 7.80 | 16.00 | 8.00 | 2.00 | 124.80 |
| Old | 4.50 | 0.25 | 5.00 | 0.05 | 1.13 |
| Total amount of recombinant IFN-α2b per plant | | | | | 137.13 |

Young leaves, top 2–3 leaves; old leaves, bottom 2–3 senescent leaves; mature leaves, selected from the middle of the plant. cpIFN-α2b levels were quantified by enzyme-linked immunosorbent assay (ELISA).

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**Table 2**

Biological activity of interferon-α2b (IFN-α2b) preparations

| Sample | Concentration (IU/mL)* |
|---|---|
| Purified cpIFN-α2b (laboratory sample) | $7.7 \times 10^7$ |
| Purified cpIFN-α2b (field sample) | $1.3 \times 10^8$ |
| cpIFN-α2b crude extract (laboratory sample) | $1.3 \times 10^6$ |
| cpIFN-α2b crude extract (field sample) | $2.8 \times 10^6$ |
| Wild-type crude extract | $4.5 \times 10^0$ |
| PEG-Intron™ (maximum weekly dose)† | $1.1 \times 10^6$ |

Baby hamster kidney (BHK) cells were pretreated with the indicated samples for 24 h prior to vesicular stomatitis virus (VSV) exposure. Concentrations were determined by comparing the results of each experimental condition with the performance of PEG-Intron™, the positive control. These data represent the mean of four independent experiments, with each treatment performed in duplicate.

* IU: International units, calculated by comparison with a leucocyte reference standard obtained from the National Institute of Allergy and Infectious Diseases (NAID) Repository.

† The maximum weekly dose is based on the manufacturer's specified dosage and is calculated for a 70-kg individual.