Transgenic Research (2005) 14:877–885
DOI 10.1007/s11248-005-1464-8
© Springer 2005

Expression of functional interleukin-12 from mouse in transgenic tomato plants

Abel Gutiérrez-Ortega[1], Claudia Sandoval-Montes[2], Teresa de Jesús Olivera-Flores[3], Leopoldo Santos-Argumedo[2] & Miguel Ángel Gómez-Lim[1,*]
[1]*Departamento de Ingeniería Genética de Plantas, Centro de Investigación y de Estudios Avanzados del I.P.N., Unidad Irapuato. Km 9.6 Libramiento Norte Carretera Irapuato-León, Apdo., Postal 629,36500, Irapuato, Guanajuato, México*
[2]*Departamento de Biomedicina Molecular, Centro de Investigación y de Estudios Avanzados del I.P.N., Av. I.P.N. 2508, 07360, Col. San Pedro Zacatenco, D.F., México*
[3]*Facultad de Química, Conjunto E, Laboratorio 116, Paseo de la Investigación Científica, Universidad Nacional Autónoma de México, 04510, DF.CP, México*

Received 25 April 2005; accepted 28 July 2005

Key words: interferon gamma, interleukin-12, recombinant proteins, tomato, transgenic plants

Abstract

Transgenic plants have been employed successfully as a low-cost system for the production of therapeutically valuable proteins, including antibodies, antigens and hormones. Here, we report the expression of a cytokine with immunomodulatory function, mouse interleukin-12 (IL-12), in transgenic tomato plants. Single-chain mouse IL-12 driven by the CaMV 35S promoter, accumulates to high levels in leaves and fruits (up to 7.3 and 3.4 μg per gram of fresh weight, respectively). Mouse IL-12 expressed in tomato displays biological activity *in vitro*, as determined by interferon-γ (IFN-γ) secretion by T cells. Possible uses of this plant-based cytokine involving mucosal delivery are discussed.

Introduction

The potential use of transgenic plants as bioreactors is now well established. Plants offer several advantages in comparison to other expression systems, including low-cost inputs, feasibility of scaling-up, reduction of health risks deriving from contamination with human pathogens, simplification of downstream or upstream processing and, most importantly, plant cells are able to perform complex posttranslational modifications (Daniell et al., 2001; Walmsley & Arntzen, 2003).

A wide variety of pharmaceutically valuable proteins has been produced in plants and they fall into three major categories: antibodies (Vaquero et al., 1999; Stoger et al., 2000), antigens (Chikwamba et al., 2002; Jani et al., 2002; Bouche et al., 2003) and biopharmaceuticals that fully retain their activity, as demonstrated by Magnuson et al. (1998), Mor et al. (2001), Zhang et al. (2003), among others. However, with a few exceptions, most biopharmaceuticals have been expressed in tobacco. This represents an obstacle in cases where therapeutic proteins are administered as crude extracts by mucosal routes, because of the presence in tobacco plants of alkaloids and other compounds toxic to humans.

Interleukin-12 (IL-12), a member of the heterodimeric cytokine family, is a 74 kDa glycoprotein composed of a 35 kDa and a 40 kDa subunit, now called α- and β-chain, respectively, linked by a disulfide bridge. IL-12 was identified as natural killer cell stimulatory factor by Kobayashi

*Author for correspondence
E-mail: mgomez@ira.cinvestav.mx

et al. (1989) and cytotoxic lymphocyte maturation factor by Stern et al. (1990) and is considered to play a central role in the interaction between the innate and adaptive arms of immunity (Trinchieri, 2003). Its effects include promotion of Th1−type cytokine response (Hsieh et al., 1993; Manetti et al., 1993), enhancement of cytotoxic responses, induction of IFN-γ, IL-2 and tumor necrosis factor (TNF)-α (Trinchieri, 2003). Furthermore, IL-12 elicits significant anti-angiogenesis and anti-tumor effects (Yao et al., 2000). IL-12 has also been used as an adjuvant when co-administered with several antigens to enhance systemic and mucosal immunity against intracellular pathogens (Boyaca et al., 1999; Gherardi et al., 1999; Jiang et al., 1999b; Palendira et al., 2002).

IL-12 chains are encoded by non-related genes (Gubler et al., 1991; Podlaski et al., 1992), whose expression is independently regulated at the transcriptional level (Carra et al., 2000). Free β-chain is produced in large excess over the IL-12 heterodimer, both as a monomer and as a homodimer, which has the ability to block IL-12 functions *in vitro* and *in vivo* (Ling et al., 1995; Heinzel et al., 1997). The creation of a single-chain polypeptide by fusion of both IL-12 chains with a linker for therapeutic use, has allowed the synthesis and production of active IL-12 (Lieschke et al., 1997).

Here we report the transformation of an edible crop, the tomato, with a construct bearing a single-chain murine IL-12 (mIL-12) gene. IL-12 is expressed in leaves and fruit and retains its biological activity in both tissues. This opens the possibility of employing fruit crude extracts without further purification for mucosal administration of IL-12.

Materials and methods

IL-12 vector construction and tomato transformation

A *Nco*I/*Bgl*II fragment corresponding to the murine IL-12 fusion gene (Jiang et al., 1999a) was cloned into pMECA (Thomson & Parrot, 1998). A *Kpn*I/*Xba*I fragment containing the IL-12 fusion gene was inserted between the CaMV 35S promoter and the 3′ UTR of CaMV 35S. The resulting plasmid was designated p35SMIL12. The expression cassette was excised with *Hin*dIII and inserted into the binary Ti plasmid pCAMBIA 2300 to obtain the p35SMIL12-2300 plasmid (Figure 1a). NPTII was employed as the selective marker. The p35SMIL12-2300 construct was transferred by electroporation into *Agrobacterium tumefaciens* strain LBA4404, which was subsequently employed for transformation of *Lycopersicon esculentum* cultivar Tanksley as previously described (Frary & Earle, 1996), with only two modifications: acetosyringone was added to the *Agrobacterium* culture medium to a final

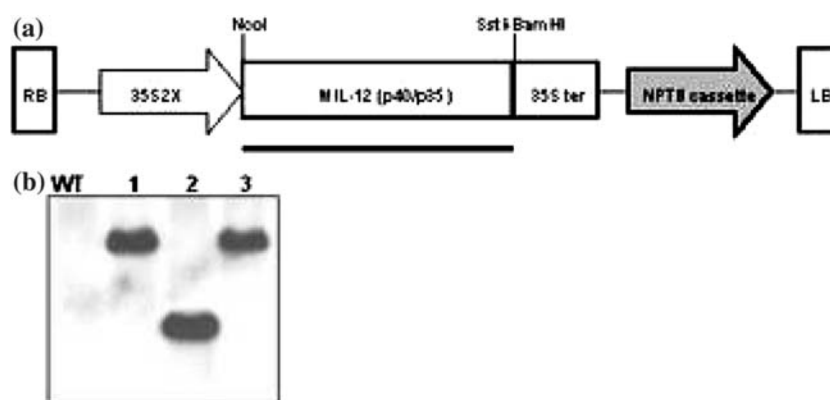

Figure 1. (a) Map showing the Ti region of plasmid p35SMIL12-2300, which was used to generate transgenic tomato plants. Mouse IL-12 expression was driven by the enhanced double CaMV 35S promoter (35S2X). The bold line represents the fragment employed as probe for both Southern and northern blot analysis. Left and right borders are indicated as LB, RB, respectively. (b) Southern blot analysis of the transgenic lines. Genomic DNA isolated from the lines was digested with *Bam*HI, fractionated by gel electrophoresis, transferred to nylon membrane, and hybridized with the radiolabeled probe. WT: non-transformed wild-type tomato plant.

concentration of 100 μM and co-culture took place at 19°C for two days in the dark.

Putative transformants selected by kanamycin resistance were transferred to soil and grown in the greenhouse. Subsequently, these plants were screened for their NPTII content with the NPTII ELISA Kit (Agdia) according to the manufacturer's protocol. Seeds from selected plants were collected, surface sterilized with 20% commercial bleach and 0.1% Tween-20 for 15 min and germinated on MS medium supplemented with 100 mg l^{-1} kanamycin. Transformed plantlets (T1) were grown in the greenhouse and utilized for further experiments.

Southern and northern blot analysis

For Southern analysis, genomic DNA was isolated from young leaves according to Wilke (1996). Twenty micrograms of DNA were digested with *Bam*HI and resolved by agarose electrophoresis and blotted to Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech) as described by Sambrook et al. (1989). The membrane was hybridized to a radioactively labeled probe synthesized with the Rediprime II kit (Amersham Pharmacia Biotech) using as template a *Nco*I/*Sst*I fragment excised from the p35SMIL12 plasmid, corresponding to the murine IL–12 fusion gene (Figure 1a). Hybridization was performed in the Rapid-hyb buffer (Amersham Pharmacia Biotech) and the membrane was exposed to Kodak Biomax Film (Kodak) at −70°C. For northern analysis, leaf and fruit RNA was extracted with the Concert Plant RNA Reagent (Invitrogen) and fractionated by denaturing formaldehyde gel electrophoresis (Sambrook et al., 1989). Blotting, hybridization and exposure were done as described above. The probe was the same as that used for Southern analysis. After northern hybridization, the probe was removed and the membrane was hybridized to a ribosomal 28S probe as loading control.

Sample preparation, western blot analysis and ELISA assay

Tomato fruits were ground in liquid nitrogen and homogenized in 4 volumes of ice-cold extraction buffer (0.01 M Na_2HPO_4, 0.003 M KH_2PO_4, 0.1 M NaCl and 0.025 M sodium ascorbate). Cleared supernatants were collected after centrifugation at 13,000 rpm for 10 min at 4°C and employed for western blot analysis and mouse IL-12 determination. Forty micrograms of total soluble protein aliquots, as determined by Bradford (1976), were lyophilized, resuspended in loading buffer (0.1 M Tris pH 8.0, 2% SDS and 0.75 M β-mercaptoethanol), boiled for 5 min, resolved by 7.5% SDS-PAGE and electrotransferred onto polyvinylidene difluoride (PVDF) membrane. The membrane was subsequently blocked with 8% non-fat milk, 0.1% Tween-20 in TBS for 1 h at room temperature. Biotinylated rat anti-mouse IL-12 monoclonal antibody C17.8 (BD Pharmingen) at 1:1000 and avidine-horseradish peroxidase conjugate (BD Pharmingen) at 1:2500 were used for IL-12 detection, which was carried out by chemiluminescence (ECL Reagent, Amersham Pharmacia Biotech). For IL-12 quantitation, cleared supernatants were assayed by sandwich ELISA, using antibodies 9A5 and C17.8 from BD Pharmingen, as capture and detection antibodies, respectively (lower limit of detection: 30 pg/ml).

For IL-12 biological activity assays, samples were prepared as follows: 10 g of leaf or fruit samples were ground in liquid nitrogen and 20 ml of cold extraction buffer (0.01 M Na_2HPO_4, 0.003 M KH_2PO_4, 0.1 M NaCl, 0.025 M sodium ascorbate, 0.5% Polyvinyl-pyrrolidone 40,000) and a plant protease inhibitor cocktail (Sigma) were added. Crude extracts were centrifuged at 10,000 rpm for 10 min at 4°C, and cleared supernatants were filtered through sieve cloth and dialyzed against PBS using 12,000–14,000 dialysis tubing (Spectra-Por). Dialyzed extracts were lyophilized, reconstituted in 2 ml of deionized water and filter-sterilized with 0.2 μm membranes (Millipore).

Biological activity assay

Biological activity was assessed by measuring production of IFN-γ by T cells (Holland et al., 1998; Losana et al., 2002). T lymphocytes were purified by panning as described by Sandoval-Montes and Santos-Argumedo (2005). Briefly, total splenocytes obtained from BALB/c mice were incubated 2 h at 37°C and 5% CO_2 in a tissue culture plate previously coated with 20 μg/ml mAb B220 (BD Pharmingen). After incubation, the unbound T lymphocytes were removed and the

procedure was repeated once again with a new plate. The T cells obtained after the two rounds of panning were cultured in RPMI 1640 medium (Gibco) supplemented with 1% nonessential amino acids (Gibco), 5×10^{-5} M 2-mercaptoethanol (Sigma) 1 mM sodium pyruvate (Sigma), 2 mM glutamine (Sigma), and 10% (v/v) fetal calf serum (Gibco), in 24-well plates (Corning) at 1×10^{6} cells/well. The T lymphocytes were stimulated with 10 μg/ml phytohemagglutinin A (Sigma) with the addition of 5 ng/ml of recombinant mouse IL-12 (Pharmingen BD) or dilutions (1:10,000 and 1:20,000) of tomato extracts expressing mouse IL-12. As negative controls, T cells were incubated without recombinant IL-12 or with non-transformed tomato plant extracts. Where indicated, anti-mouse IL-12 antibodies (BD Pharmingen) were added at a 10 μg/ml concentration. After two days of culture, supernatants were collected and their IFN-γ concentration quantified by a sandwich ELISA. The ELISA kit was purchased from BD Pharmingen (lower limit of detection: 5 pg/ml).

Results

Tomato plant transformation with mIL-12 fusion gene construct

We transformed tomato cotyledons with the construct p35SMIL12–2300 via *Agrobacterium tumefaciens* infection. In this construct, the mIL-12 fusion gene expression was driven by the constitutive, enhanced CaMV 35S. We recovered seven plants on selective medium after transformation, from which three tested positive for NPTII (data not shown). These plants, designated as mIL12-1, -2 and -3, were grown in the greenhouse to maturity and selfed. Their progeny was employed for subsequent experiments. Constitutive expression of mIL-12 in transgenic tomatoes did not seem to cause any apparent phenotypic alteration in the plants. The plants looked normal and flowered at the same time than non-transformed plants (data not shown).

Mouse IL-12 expression at the transcriptional and translational level

Integration of mIL-12 fusion gene in the three lines was confirmed by Southern blot hybridization. We found that a single copy of the transgene was inserted in the genome of all three lines (Figure 1b). DNA from wild type plants did not hybridize with the probe.

Total RNA isolated from wild type and mIL12 lines was used to determine IL-12 mRNA expression by northern blotting. Figure 2a reveals that IL-12 mRNA is accumulated to similar levels in all lines. Also, IL-12 transcripts are present both in leaves and fruits as expected, since the promoter employed drives constitutive expression. There was no detectable signal when using RNA from wild type plants.

Western blot analysis showed a 70–75 kDa band present in fruit extracts from all lines and reacting with specific antibodies against mouse IL-12 (Figure 2b). The band was not detectable in protein extracts from non-transformed fruit. In addition, this band displays a similar molecular weight to that reported by Lieschke et al. (1997).

As determined by ELISA, IL-12 production in our lines varied from 1 to 3.4 and from 2.7 to 7.3 μg/g of fresh weight in fruit and leaves, respectively (Figure 3). When we determined by

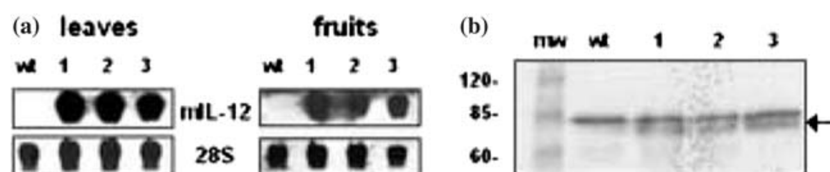

Figure 2. Mouse IL-12 expression at the transcriptional and translational level in transgenic tomato plants. (a) Northern blot analysis of the recovered tomato lines. Total RNA (20 μg) from leaves and fruits was probed with mouse IL-12 gene (mIL-12) or a ribosomal 28S gene (28S) as loading control. (b) Immunoblot analysis of fruit crude extracts from mouse IL-12 transgenic lines. Forty micrograms of total soluble protein from the transgenic lines and non-transformed tomato plant (wt) were separated by 7.5% SDS-PAGE and transferred to PVDF membrane. Biotinylated anti-mouse IL-12 monoclonal antibody and avidin-horseradish peroxidase conjugate were used for chemiluminescence detection. The sizes of the molecular weight markers (mw) are indicated on the left in kDa. The numbers at the top refer to the different tomato lines tested. wt: non-transformed, wild type plants.

ELISA the expression of IL-12 in T2 plants from one of our lines, we found that the levels in the fruit and leaves were similar to the parent plant (data not shown), indicating that expression of the transgene is stable through generations.

Biological activity of plant expressed mouse IL-12

Biological activity of mIL-12 was assessed by induction of IFN-γ secretion in T cells (Holland et al., 1998; Losana et al., 2002; Rosenzweig & Holland, 2005). Figure 4 shows that extracts from transformed tomato plants (leaf and fruit) were able to induce IFN-γ production. In addition, crude extracts from a non-transformed plant did not induce IFN-γ production. In order to confirm that the plant-derived mIL-12 was causing IFN-γ production and secretion, anti-mouse IL-12 antibodies were added together with the plant extracts. As shown in Figure 4, in the presence of the specific antibodies against mIL-12, IFN-γ production was drastically reduced.

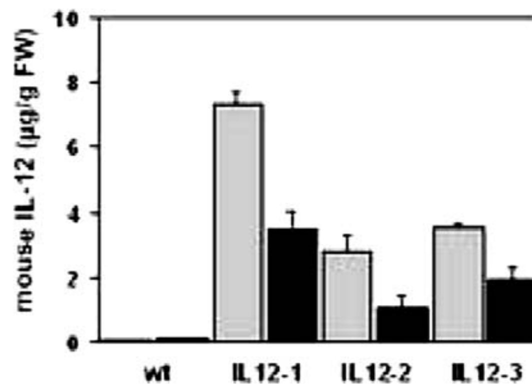

Figure 3. Quantitation of mIL-12 levels in transgenic tomato plants. Mouse IL-12 levels present in leaf (gray bars) and fruit (black bars) crude extracts from the transgenic lines were assessed by ELISA as described in the Materials and methods section. Each bar represents the mean ± SD (expressed in micrograms per gram of fresh weight) of three separate determinations for a particular line. wt: extracts from non-transformed, wild-type tomato plant.

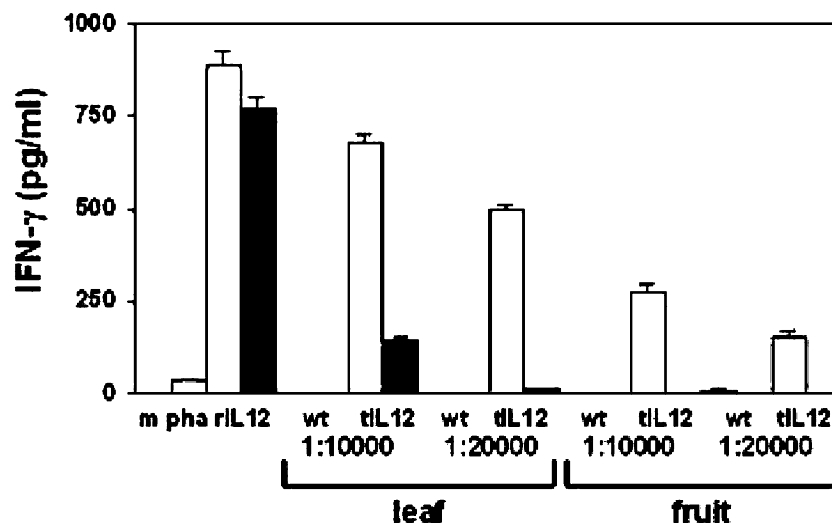

Figure 4. Bioactivity assay of the plant-derived mouse IL-12. Secretion of IFN-γ by T cells induced with recombinant mouse IL-12 (rIL12), dilutions of wild-type (wt) and transformed (tIL12) tomato extracts in the absence (white bars) or presence (black bars) of anti-mouse IL-12 antibodies. As negative controls, T cells were grown in the presence of medium alone (m) and phytohemagglutinin A (pha). The assay was performed in triplicate.

Discussion

In this work we report the expression of a key immunomodulatory protein, IL-12 from mouse, in tomato plants. Since IL-12 had been successfully administered as adjuvant via mucosal surfaces (Belyakov et al., 1998; Boyaca et al., 1999), it seemed appropriate to evaluate a plant-expressed IL-12 as adjuvant and as therapeutic agent by mucosal administration. To this aim, we had previously express human IL-12 in tobacco plants to be tested in mice and subsequently in human cells, but two factors prevented us from achieving this goal. First, the expression levels in tobacco were very low in comparison to the levels attained in this work (see below). Second, human IL-12 is not functional in mice cells. Even though there is a 60–70% homology in amino acid sequence between the murine and the human IL-12 proteins, human IL-12 is only active in human cells, whereas murine IL-12 is active in mouse and human cells (Schoenhaut et al., 1992). For these reasons, we decided to express the murine gene in an edible plant, tomato. Complementary DNAs coding for other cytokines (IL-2 and IL-4) had been expressed previously in tobacco cell suspension cultures (Magnuson et al., 1998).

Analysis of the three tomato lines obtained showed that they contained only one copy of the transgene, whereas the mRNA of mIL-12 accumulated to similar levels in leaves and fruits in all lines. A band displaying the same size as that reported in the original article of the single-chain mouse IL-12 was detectable by specific antibodies in fruit from all lines indicating successful expression in tomato. A positive control (recombinant mIL-12) could not be included in the gel, because the proteins were separated under reducing conditions, which dissociates IL-12 into the α and β subunits, and the monoclonal antibody employed reacts only with mIL-12 in its heterodimeric form. The western blot also revealed the presence of a second band just slightly above the putative mIL-12 band. We do not know the identity of this band, which is also present in the non-transformed line, but we hypothesize that it corresponds to a plant protein recognized by the antibodies. This is not an uncommon result when using antibodies raised against animal proteins for detection in plants. However, this band did not interfere with IL-12 quantitation by ELISA, because values from untransformed plants were closer to zero. This is explained by the high sensitivity of the sandwich ELISA employed, in which we used two antibodies that recognized two different epitopes of the same molecule, making the assay much more specific. Even though the putative mIL-12 appeared to accumulate to similar levels in all lines, ELISA indicated that the production was variable. We do not know the reason, but expression of an antigen in fruit and leaves of the same tomato plant can be variable (Walmsley et al., 2003).

The levels of expression obtained are within the range of expression of other biopharmaceuticals that have been expressed in plants before (Daniell et al., 2001). However, these values were much higher than those that we had previously obtained for human IL-12 expressed in tobacco plants (Gutiérrez-Ortega et al., 2004). This was somewhat surprising if one considers that both the human and murine genes were driven by the same promoter, the enhanced 35S CaMV. There is a high degree of homology between human and murine IL-12 and they both cross-hybridize, however, this does not necessarily mean that they should perform similarly as indicated by the fact the human IL 12 is *not* active in murine cells, whereas murine IL-12 is active in human cells. In addition, a different plant system was employed in either case and the two genes, although similar in function, were of different origin. All these facts might explain the difference in expression level. We employed the native genes in both cases and thus they were not optimized for plant expression. Perhaps there were differences in translatability of both genes in the plant cells, something that may have also contributed to the difference in expression.

The plant-produced mIL-12 was able to induce the secretion of IFN-γ by T cells, which is a conventional assay to evaluate IL-12 activity. This effect was obtained by using crude extracts from fruits, and no purification of the cytokine was necessary. Increasing amounts of the plant-based mIL-12 induced the secretion of higher amounts of IFN-γ as expected. Incubation of mIL-12 with the monoclonal antibodies inhibited activity, which demonstrated that IFN-γ induced by *bona fide* mIL-12 produced by the plant. It should be noted, however, that in the lower dilution of transformed leaf extract, IFN-γ production was not fully abolished in the presence of antibodies. This might have

been caused by the use of insufficient antibody. Further experiments to compare the specific activity of this plant-produced molecule with the commercially available cytokine need to be carried out.

As mentioned earlier, native mIL-12 is a glycoprotein. Several studies had demonstrated that plant recombinant glycoproteins are present in multiple glycoforms (Magnuson et al., 1998; James et al., 2000). This is what we had detected previously with human IL-12 in which more than one band was detected in transgenic tobacco by western blot (Gutiérrez-Ortega et al., 2004). Apparently, no different mouse IL-12 glycoforms were generated in our transgenic tomato lines. Glycosylation in plants differs slightly from that in mammals (Bardor et al., 1999) and these differences may affect bioactivity, as is the case of erythropoietin expressed in tobacco cells (Matsumoto et al., 1995). Glycosylation is essential for secretion of α- and β-IL-12 chains (Carra et al., 2000; Murphy et al., 2000; Ha et al., 2002), but there are no data linking IL-12 glycosylation with bioactivity. However, a more detailed characterization needs to be carried out of bioactivity of tomato-derived IL-12 in comparison to commercial IL-12. Further biochemical characterization, mainly focused on the extent of glycosylation, is also needed.

Since IL-12, (human and murine), is composed of two separate chains encoded by different genes, several strategies to deliver equal amounts of both chains, an important requirement for activity, have been designed. Our results clearly indicate that synthesis of a single-chain polypeptide fused by a linker sequence is an excellent option to produce a functional IL-12 in heterologous systems such as plants.

IL-12 has been administered effectively via mucosal routes in previous works. It will be very interesting to test the efficacy of the plant-produced mIL-12 as an adjuvant in oral immunization with different antigens. An important finding in this respect is that mucosal (intranasal) delivery of IL-12 reduces toxicity without altering its efficacy (Huber et al., 2003). Preliminary results using plant-based mIL-12 in a tuberculosis-mouse model indicate that IL-12 present in these extracts displays biological activity *in vivo* when delivered intranasally (Gutierrez-Ortega, A., Hernandez-Pando, R, and Gómez Lim, M.A., unpublished results).

Acknowledgements

We thankfully acknowledge the technical assistance of Luis Jorge Saucedo-Arias, M.Sc. Hector Romero-Ramirez and MVZ Ricardo Gaxiola-Centeno. This research was partially supported by a CONACyT Grant (#28772-N) to M.A.G.L. We are indebted to CONACYT for a PhD scholarship to A.G.O. (#128924). We thank Dr. C. Jiang for the kind gift of the mIL-12 fusion gene.

References

Bardor M, Faye L and Lerouge P (1999) Analysis of the *N*-glycosylation of recombinant glycoproteins produced in transgenic plants. *Trends Plant Sci* **4**: 376–380.

Belyakov IM, Ahlers JD, Brandwein BY, Earl P, Kellsall BL, Moss B, Strober W and Berzofsky JA (1998) The importance of local mucosal HIV-specific $CD8^+$ cytotoxic T lymphocytes for resistance to mucosal viral transmission in mice and enhancement of resistance by local administration of IL-12. *J Clin Invest* **102**: 2072–2081.

Bouche FB, Marquet-Blouin E, Yanagi Y, Steinmetz A and Muller CP (2003) Neutralising immunogenicity of a polyepitope antigen expressed in a transgenic food plant: a novel antigen to protect against measles. *Vaccine* **21**: 2065–2072.

Boyaca PN, Marinaro M, Jackson RL, Menon S, Kiyono H, Jirillo E and McGhee JR (1999) IL-12 is an effective adjuvant for induction of mucosal immunity. *J Immunol* **162**: 122–128.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254.

Carra G, Gerosa F and Trinchieri G (2000) Biosynthesis and posttranslational regulation of human IL-12. *J Immunol* **164**: 4752–4761.

Chikwamba RK, Cunnick J, Hathaway D, McMurray J, Mason H and Wang K (2002) A functional antigen in a practical crop: LT-B-producing maize protects mice against *Escherichia coli* heat labile enterotoxin (LT) and cholera toxin (CT). *Transgenic Res* **11**: 479–493.

Daniell H, Streatfield SJ and Wycoff K (2001) Medical molecular pharming: production of antibodies, biopharmaceuticals and edible vaccines in plants. *Trends Plant Sci* **6**: 219–226.

Frary A and Earle ED (1996) An examination of factors affecting the efficiency of *Agrobacterium*-mediated transformation of tomato. *Plant Cell Rep* **16**: 235–240.

Gherardi MM, Ramirez JC, Rodriguez D, Rodriguez JR, Ichio G, Zavala F and Esteban M (1999) IL-12 delivery from recombinant vaccinia virus attenuates vector infectivity and enhances cellular immune response against HIV-1 gp160 protein in a dose-dependent manner. *J Immunol* **162**: 6724–6733.

Gubler U, Chua AO, Schoenhaut DS, Dwyer CM, McComas W, Motyka R, Nabavi N, Wolitzky AG, Quinn PM, Familletti PC and Gately MK (1991) Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proc Natl Acad Sci USA* **88**: 4143–4147.

Gutiérrez-Ortega A, Avila-Moreno F, Saucedo-Arias LJ, Sanchez-Torres C and Gómez-Lim MA (2004) Expression of a single-chain human interleukin-12 gene in transgenic

tobacco plants and functional studies. *Biotechnol Bioeng* **85**: 734–740.
Ha SJ, Chang J, Song MK, Su YS, Jin HT, Lee CH, Nam GH, Choi G, Choi KY, Lee SH, Kim WB and Sung YC (2002) Engineering N-glycosylation mutations in IL-12 enhances sustained cytotoxic T lymphocyte responses for DNA immunization. *Nat Biotechnol* **20**: 381–386.
Heinzel FP, Hujer AM, Ahmed FN and Rerko RM (1997) In vivo production and function of IL-12 p40 homodimers. *J Immunol* **158**: 4381–4388.
Holland SM, Dorman SE, Kwon A, Pitha-Rowe IF, Frucht DM, Gerstberger SM, Noel GJ, Vesterhus P, Brown MR and Fleisher TA (1998) Abnormal regulation of interferon-gamma, interleukin-12, and tumor necrosis factor-alpha in human interferon-gamma receptor 1 deficiency. *J Infect Dis* **178**: 1095–1104.
Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A and Murphy KM (1993) Development of T_H1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* **260**: 547–549.
Huber VC, Arulanandam BP, Arnaboldi PM, Elmore MK, Sheehan CE, Kallakury BVS and Metzger DW (2003) Delivery of IL-12 intranasally leads to reduced IL-12-mediated toxicity. *Int Immunopharmacol* **3**: 801–809.
James EA, Wang C, Wang Z, Reeves R, Shin JH, Magnuson NS and Lee JM (2000) Production and characterization of biologically active human GM-CSF secreted by genetically modified plant cells. *Protein Expr Purif* **19**: 131–138.
Jani D, Meena LS, Mamad Q, Haq R, Singh Y, Sharma AK and Tyagi AK (2002) Expression of cholera toxin B subunit in transgenic tomato plants. *Transgenic Res* **11**: 447–454.
Jiang C, Magee DM and Cox R (1999a) Construction of a single-chain interleukin-12-expressing retroviral vector and its application in cytokine gene therapy against experimental coccidiomycosis. *Infect Immun* **67**: 2996–3001.
Jiang C, Magee DM and Cox R (1999b) Coadministration of interleukin 12 expression vector with antigen 2 cDNA enhances induction of protective immunity against *Coccidioides immitis*. *Infect Immun* **67**: 5848–5853.
Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, Loudon R, Sherman F, Perusia B and Trinchieri G (1989) Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J Exp Med* **170**: 827–845.
Lieschke GJ, Rao PK, Gately MK and Mulligan RC (1997) Bioactive murine and human interleukin-12 fusion proteins which retain antitumor activity *in vivo*. *Nat Biotechnol* **15**: 35–40.
Ling P, Gately MK, Gubler U, Stern AS, Lin P, Hollfelder K, Su C, Pan YC and Hakimi J (1995) Human IL-12 p40 homodimer binds to the IL-12 receptor but does not mediate biologic activity. *J Immunol* **154**: 116–127.
Losana G, Rigamonti L, Borghi I, Assenzio B, Ariotti S, Jouanguy E, Altare F, Forni G, Casanova JL and Novelli F (2002) Requirement for both IL-12 and IFN-gamma signaling pathways in optimal IFN-gamma production by human T cells. *Eur J Immunol* **32**: 693–700.
Magnuson NS, Linzmaier PM, Reeves R, An G, HayGlass K and Lee JM (1998) Secretion of biologically active human interleukin-2 and interleukin-4 from genetically modified tobacco cells in suspension culture. *Protein Expr Purif* **13**: 45–52.
Manetti R, Parronchi P, Giudizi MG, Piccinni MP, Maggi E, Trinchieri G and Romagnani S (1993) Natural killer cell stimulatory factor (interleukin 12[IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J Exp Med* **177**: 1199–1204.
Matsumoto S, Ikura K, Ueda M and Sasaki R (1995) Characterization of a human glycoprotein (erythropoietin) produced in cultured tobacco cells. *Plant Mol Biol* **27**: 1163–1172.
Mor TS, Sternfeld M, Soreq H, Arntzen CJ and Mason HS (2001) Expression of recombinant human acetylcholinesterase in transgenic tomato plants. *Biotechnol Bioeng* **75**: 259–266.
Murphy FJ, Hayes MP and Burd PR (2000) Disparate intracellular processing of human IL-12 preprotein subunits: atypical processing of the P35 signal peptide. *J Immunol* **164**: 839–847.
Palendira U, Kamath AT, Feng CG, Martin E, Chaplin PJ, Triccas JA and Britton WJ (2002) Coexpression of interleukin-12 chains by a self-splicing vector increases the protective cellular immune response of DNA and *Mycobacterium bovis* BCG vaccines against *Mycobacterium tuberculosis*. *Infect Immun* **70**: 1949–1956.
Podlaski FJ, Nanduri VB, Hulmes JD, Pan YE, Levin W, Danho W, Chizzonite R, Gately MK and Stern AS (1992) Molecular characterization of interleukin 12. *Arch Biochem Biophys* **294**: 230–237.
Rosenzweig SD and Holland SM (2005) Defects in the interferon-gamma and interleukin-12 pathways. *Immunol Rev* **203**: 38–47.
Sambrook J, Fritsch EF and Maniatis T (1989) Molecular Cloning: A Laboratory Manual, 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sandoval-Montes C and Santos-Argumedo L (2005) CD38 is expressed selectively during the activation of a subset of mature T cells with reduced proliferation but improved potential to produce cytokines. *J Leuk Biol* **77**: 513–521.
Schoenhaut DS, Chua AO, Wolitzky AG, Quinn PM, Dwyer CM, McComas W, Familletti PC, Gately MK and Gubler U (1992) Cloning and expression of murine IL-12. *J Immunol* **148**: 3433–3440.
Stern AS, Podlaski FJ, Hulmes JD, Pan YCE, Quinn PM, Wolitzky AG, Familletti PC, Stremlo DL, Truitt T, Chizzonite R and Gately MK (1990) Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. *Proc Natl Acad Sci USA* **87**: 6808–6812.
Stoger E, Vaquero C, Torres E, Sack M, Nicholson L, Drossard J, Williams S, Keen D, Perrin Y, Christou P and Fischer R (2000) Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. *Plant Mol Biol* **42**: 583–590.
Thomson JM and Parrot WA (1998) pMECA: a cloning plasmid with 44 unique restriction sites that allows selection of recombinants based on colony size. *Biotechniques* **24**: 922–924.
Trinchieri G (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* **3**: 133–146.
Vaquero C, Sack M, Chandler J, Drossard J, Schuster F, Monecke M, Schillberg S and Fischer R (1999) Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves. *Proc Natl Acad Sci USA* **96**: 11128–11133.
Walmsley AM and Arntzen CJ (2003) Plant cell factories and mucosal vaccines. *Curr Opin Biotechnol* **14**: 145–150.
Walmsley AM, Alvarez ML, Jin Y, Kirk DD, Lee SM, Pinkhasov J, Rigano MM, Arntzen CJ and Mason HS (2003)

Expression of the B subunit of *Escherichia coli* heat-labile enterotoxin as a fusion protein in transgenic tomato. *Plant Cell Rep* **21**: 1020–1026.

Wilke S (1996) Isolation of total genomic DNA. In: Clark MS (ed), Plant Molecular Biology. (pp. 3–15) Springer-Verlag, Berlin.

Yao L, Pike SE, Setsuda J, Parekh J, Gupta G, Raffeld M, Jaffe ES and Tosato G (2000) Effective targeting of tumor vasculature by the angiogenesis inhibitors vasostatin and interleukin-12. *Blood* **96**: 1900–1905.

Zhang B, Yang YH, Lin YM, Rao Q, Zheng GG and Wu KF (2003) Expression and production of bioactive human interleukin-18 in transgenic tobacco plants. *Biotechnol Lett* **25**: 1629–1635.