

Expression of active human epidermal growth factor (hEGF) in tobacco plants by integrative and non-integrative systems

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Abstract

Human epidermal growth factor (hEGF) was expressed in *Nicotiana* plants using integrative and non-integrative systems. Nuclear transformation was carried out using genetic constructs that allow hEGF accumulation into the cytoplasmic or apoplastic spaces. Protein level did not exceed 0.00001% of total soluble protein in the cytoplasm, but reached values of up to 0.11% when hEGF was targeted to the apoplast. In addition, cytoplasmic and apoplastic hEGF versions were cloned into a viral vector derived from potato virus X. Transcripts from recombinant viruses were used to infect *Nicotiana benthamiana* and *Nicotiana tabacum* plants. While the recombinant protein was barely detectable in the case of the cytoplasmic version, it reached levels of up to 0.015% of total soluble proteins in plants infected with the apoplastic version. Extracts from transgenic plants exhibiting the highest hEGF accumulation and from plants infected with the viral apoplastic version were tested for biological activity in cumulus cells expansion assays. hEGF containing extracts showed biological activity comparable to commercial hEGF. Finally, radioreceptor binding assays showed that tobacco-expressed hEGF binds to its receptor with the same affinity as commercial hEGF. These data suggest that hEGF accumulation can be significantly increased by implementing adequate exporting strategies.

Abbreviations: hEGF – human epidermal growth factor; PVX – potato virus X; TMV – tobacco mosaic virus; CaMV – cauliflower mosaic virus

Introduction

Expression of biopharmaceutical proteins in transgenic plants is an attractive alternative due to the possibility of reducing production costs and to overcoming the limitations of other expression systems. Large-scale production of eukaryotic proteins is feasible in bacterial cultures at relatively low cost, but many post-translational modifications needed for bio-

logical activity do not occur in prokaryotic cells, making them unusable to this purpose. In addition, expression of foreign proteins in bacteria frequently results in intracellular aggregates because of incorrect folding or lack of disulfide bridges. These aggregates are often difficult to dissolve, lowering protein yields and increasing purification costs. Alternatively, most proteins produced in transgenic animals or mammalian cell cultures are identical to those from their

natural counterparts, but bulk production costs are usually much higher. For example, whereas IgA production costs have been estimated between 100-1000 US\$/g of protein in mammalian cell cultures or transgenic goats, they were estimated to be less than 50 US\$/g in transgenic plants (Daniell et al. 2001; Doran 2000). A major advantage of plants is that scaling-up is relatively inexpensive as compared to other expression systems, especially in those crops in which harvesting and processing are based on well-established practices. Accumulation of heterologous proteins in storage organs such as seeds, tubers and roots could greatly reduce the amount of biomass to be processed and further facilitate extraction procedures (Fischer et al. 1999; Giddings et al. 2000; Daniell et al. 2001). An additional advantage of plant systems is the absence of contamination with animal pathogens potentially associated with human health hazards, such as viruses or prions.

In many cases, attempts to express useful proteins by transforming plant nuclear genomes resulted in accumulation levels that were insufficient for practical application. For example, human serum albumin was expressed in potato plants to a level of 0.020% of the total soluble protein (Sijmons et al. 1990). Similarly, bovine aprotinin and human erythropoietin were expressed in tobacco at levels of 0.069% and 0.0026%, respectively (Zhong et al. 1999; Matsumoto et al. 1995). Recombinant proteins can be targeted to storage organs which would allow stable accumulation and storage. Thus, avidin expression in maize seeds allowed transgenic protein accumulation up to 2% of aqueous extractable protein and commercial exploitation of this protein (Hood et al. 1997). High accumulation levels have also been reported for different classes of human antibodies and antibody-derived molecules (Daniell et al. 2001).

High accumulation levels are a critical factor to enable commercial production of human proteins in plants. Gene expression can be optimized by using strong promoters, improving transcript RNA stability, enhancing translation of transgenic mRNAs or optimizing codon usage (Perlak et al. 1991). Stability of recombinant proteins is also dependent on the cellular environment and the presence of appropriate chaperone proteins; therefore, sub-cellular targeting is an important consideration that must be borne in mind when planning the production of a candidate protein (Stoger et al. 2002).

In past work, Higo et al. (1993) expressed a synthetic hEGF gene in transgenic tobacco plants and re-

ported protein accumulation levels of up to 0.001% of total soluble protein. In this work, using the same plant system but different genetic constructs, we expressed hEGF at levels of up to 0.11% of total soluble proteins when the protein was directed to the apoplastic space. Improved accumulation was also obtained when an apoplastic hEGF version was transiently expressed from a viral vector based in the potato virus X (PVX) genome. It was also shown that extracts obtained from transgenic or recombinant virus-infected plants promote the same kind of morphological changes as commercial hEGF when tested in cumulus cell expansion assays. Additionally, tobacco expressed-hEGF and commercial hEGF showed comparable behavior in radioligand assays.

Materials and methods

Genetic constructs

Plasmid pETEGF, containing a synthetic version of the hEGF gene, was kindly provided by Dr. Gerardo Glikin (BioSidus S.A., Argentina). Three different constructs were generated in order to integrate the hEGF sequence into the nuclear genome of tobacco plants (Figure 1A, B). The p35EGF plasmid was obtained by cloning the hEGF sequence into the Xba I and Sac I sites of binary vector pBI121 (Clontech, USA), under the transcriptional control of the cauliflower mosaic virus (CaMV) 35S promoter and the *Agrobacterium tumefaciens* nopaline synthase transcription termination sequence. Plasmid p35(L)EGF was constructed by replacing the CaMV 35S promoter with a version extended up to 1340 bp from the transcription start (Coego et al. 1996), followed by the Ω translational enhancer of tobacco mosaic virus (TMV). The CaMV 35S long promoter and the Ω sequence were subcloned from plasmid pHAP12, kindly provided by Dr. Lázaro Hernández (CIGB, Cuba). Both p35EGF and p35(L)EGF allow hEGF accumulation in the cytoplasm and are therefore referred as 'cytoplasmic' versions. The third construct allows expression of an N-terminal fusion comprising the sequences coding for the signal peptide of the tobacco AP24 osmotin (the first 22 codons of AP24) and the complete hEGF sequence. To facilitate cloning, two Eco RI restriction sites were created by silent mutagenesis in both the hEGF 5' region and in the AP24 region flanking the signal peptide processing site. DNA fragments containing the AP24 and

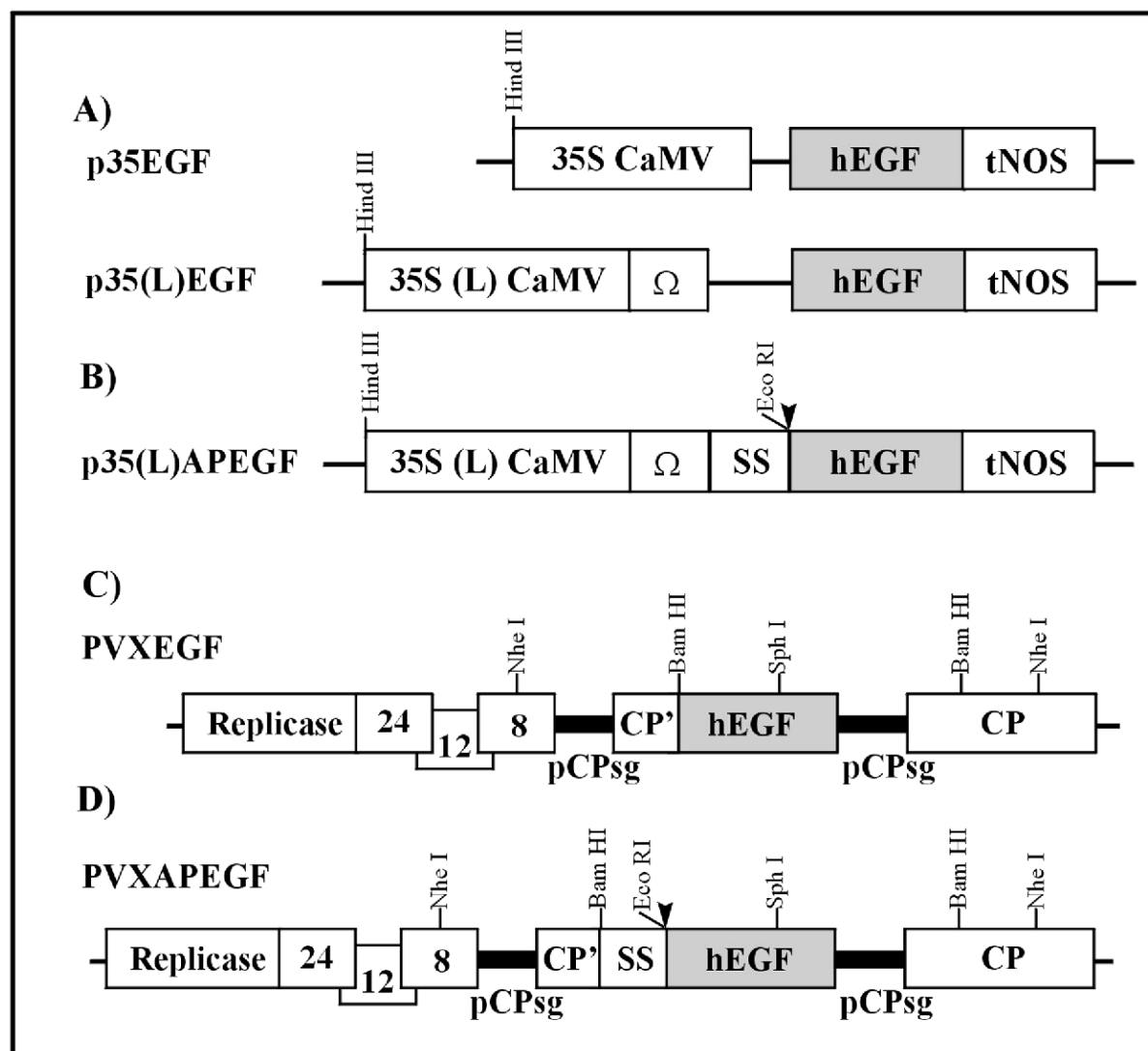


Figure 1. Expression cassettes used for *N. tabacum* transformation. A) Schematic representations of the genetic constructs allowing cytoplasmic hEGF accumulation under the control of the CaMV 35S promoter (p35EGF) or the long CaMV 35S promoter and the TMV Ω enhancer (p35(L)EGF). B) Schematic representation of the genetic construct allowing apoplastic hEGF accumulation under the transcriptional control of the long CaMV 35S promoter and the TMV Ω enhancer (p35(L)APEGF). C) Representation of the viral vector allowing cytoplasmic expression of hEGF (PVXEGF). The hEGF sequence was fused to the first 14 amino acids of the PVX coat protein (CP') under the transcriptional control of a duplicated PVX coat protein subgenomic promoter (pCPsg). D) Representation of the viral vector allowing apoplastic expression of hEGF (PVXAPEGF). The hEGF sequence was fused to the AP24 signal sequence (SS) under the transcriptional control of the duplicated PVX coat protein subgenomic promoter; SS: extracellular export sequence from AP24 osmotin; tNOS: nopaline synthase terminator sequence; Ω: translational enhancer sequence. Replicase: PVX RNA-dependent RNA-polymerase; 24, 12, 8: PVX triple gene block; CP: coat protein. The arrowhead indicates the signal sequence processing site.

hEGF sequences were obtained by PCR amplification using the oligonucleotide primers 5'-CTCGGAAT-TCGCAGCATAAG-3' and 5'-ATCTCCACTGACG-TAAGGGA-3', for AP24, and 5'-ATGAATTCCGAT-TCTGAATG – 3' and 5'-GATTTAGGTGACACTA-TAG-3', for hEGF, in which the Eco RI restriction

sites are shown underlined. The PCR amplification products were purified and digested with Sac I and Eco RI, in the case of AP24, and with Eco RI and Hind III, in the case of hEGF, and cloned into the plasmid pGEM3Zf+ (Promega, USA) to create plasmid pGEMAPEGF. After DNA sequencing to verify

proper in-frame fusion, the resulting chimeric EGF sequence was cloned under the control CaMV 35S long promoter and the TMV Ω sequence to obtain plasmid p35(L)APEGF. In the final construct, two alanine residues are conserved in the flanks of the processing site, in such a way that, after cleavage of the signal peptide between residues 21 and 22 of AP24, the released hEGF molecule has an alanine at its N-terminus. p35(L)APEGF is referred as the 'apoplastic' version. The tobacco AP24 sequence was amplified from plasmid pHAP12 (Dr. Lázaro Hernández, CIGB, Cuba).

Viral vector constructs:

A full-length copy of the PVX genome (strain CP; Orman et al. 1990) was obtained by assembling appropriate cDNA fragments. The resulting clone, named pPVX3, encompassing nucleotides 1-6432 and a poly(A) tail of 24 residues, was ligated downstream of the T7 polymerase promoter and introduced into the pUC18 *E. coli* vector (New England Biolabs, USA). Plasmid pPVX3 was used to obtain a gene insertion vector named pPVX-GUS. In this construct, the *gus-A* gene is expressed under the control of a duplicated coat-protein subgenomic promoter (Calamante 1998). The pPVXEGF version was obtained by further modification of the pPVXGUS sequence. To this aim, a plasmid containing a Nhe I/Nhe I fragment from pPVX-GUS – corresponding to positions 4874-5792 of the PVX genome – was double-digested with Sma I/Stu I to remove the *gus-A* gene and the hEGF sequence – obtained as a Nde I Klenow blunt ended/Sma I fragment – was ligated into the same sites to form plasmid pBSXEGF. Then, the Nhe I fragment was removed from pBSXEGF and re-introduced into pPVX-GUS replacing the original sequence to create plasmid pPVXEGF (Figure 1C). In this construct, hEGF is expressed as a C-terminal fusion protein to the first 14 amino acids of the PVX coat protein which is targeted to the cell cytoplasm. A second version of the viral vector, allowing hEGF accumulation into the apoplastic space, was obtained by replacing a Bam HI/Sph I hEGF 5' fragment from pPVXEGF with the corresponding sequence from pGEMAPEGF. This version was named pPVXAPEGF (Figure 1D).

Plant transformation

Constructs p35EGF, p35(L)EGF and p35(L)APEGF were introduced into *Agrobacterium* strain LBA 4404

by the 'freeze and thaw' method described by Holsters et al. (1978). Transformation into *N. tabacum* cv Xanthi D8 via *A. tumefaciens* was performed using leaf disks as explants (Horsch et al. 1985). Transformed plants were isolated on medium containing kanamycin as a selection agent.

Infection assays in N. benthamiana and N. tabacum plants

Viral RNA transcripts were *in vitro* synthesized from linearized plasmids pPVX3, pPVXEGF or pPVXAPEGF following the protocol provided with the *RiboMAX T7* RNA transcription kit (Promega, USA) and adding 10% v/v DMSO and 0.6 mM Cap analog (m⁷GpppG; New England Biolabs, USA) to the reaction mixture. Each infection assay included 5 to 8 *N. benthamiana* plants and was repeated at least 3 times. Eleven to 13 weeks after seed germination, the third leaves from the top were mechanically inoculated with 1-2 µg of viral RNA using carborundum powder (Fisher Scientific USA) as an abrasive. Leaves were then rinsed with tap water and plants were kept in a growth chamber under a 16h/8h light/dark photoperiod. Symptoms of infection were visible after 7-8 days in the form of mild chlorotic lesions. Samples were collected from the third new fully-expanded leaves by punching the tissue with the cap of a 1.5-ml Eppendorf tube at 18 and 30 days after inoculation. Three samples from different regions of the leaf were collected for each plant. Infection and sampling of *N. tabacum* Xanthi D8 plants was performed in the same way as *N. benthamiana* but using extracts from PVXAPEGF-infected *N. benthamiana* leaves as source of inoculum.

PCR analysis

Plants rooting in kanamycin-supplemented medium were tested for the presence of the transgenes by PCR amplification. Total genomic DNA from transgenic and non-transgenic (one) plants was extracted according to Dellaporta et al. (1983). Oligonucleotide primers complementary to the 3'-regions of the CaMV 35S promoter (5'-ATCTCCACTGACGTAAGGGA-3') and the hEGF gene (5'-CTCGAGTCAGCGCAGTTC-3') were used to amplify DNA fragments from different transgenic plants.

Southern blot analysis

Integration into the tobacco genome and transgene copy number were assessed by Southern blot analysis. Total DNA was extracted from leaves as described by Dellaporta et al. (1983). Aliquots of 10 µg of genomic DNA were treated with Hind III (Promega, USA). The digestion products were separated in 0.8% agarose gels and transferred onto an Immobilon N+ nylon membrane (Amersham-Pharmacia Biotech, USA). Specific DNA sequences were detected by hybridization with ³²P-labeled hEGF cDNA probes labeled by random priming with a commercial kit (Prime-a-Gene, Promega, USA). After hybridization, membranes were washed twice with gentle shaking for 30 min in 0.2X SSC, 0.1% SDS, at 65 °C

Northern blot analysis

mRNA levels in transgenic plants were analyzed by Northern blot hybridization. Total RNA was extracted from leaves as previously described (Verwoerd et al. 1989). 20 µg of RNA were separated onto a 1.5% denaturing agarose gel and blotted onto an Immobilon N+ nylon membrane (Amersham-Pharmacia Biotech, USA). Specific mRNA sequences were detected by hybridization with ³²P-labeled hEGF cDNA probes labeled by random priming with a commercial kit (Prime-a-Gene, Promega, USA). After hybridization, membranes were washed twice with gentle shaking for 30 min in 0.2X SSC, 0.1% SDS, at 65 °C.

Acid-ethanol extraction procedure

Proteins were extracted from fresh leaf tissue by the acid-ethanol procedure described by Roberts et al. (1980) with minor modifications. About 30 g of fresh leaf tissue were ground in liquid nitrogen and thawed in 40 ml/10 g of tissue of a solution containing 375 ml of 95% (v/v) ethanol, 7.5 ml of concentrated HCl and 33 mg of phenylmethylsulfonylfluoride and 1.9 mg of pepstatin as protease inhibitors. Volume was adjusted to 6 ml per g of tissue with distilled water and extraction was left to proceed at 4 °C overnight with gentle agitation. The solution was centrifuged at 6,000 g for 30 min and the supernatant was then brought to pH 5.2 with concentrated ammonium hydroxide, followed by addition of 1 ml of 2 M ammonium acetate buffer, pH 5.3, for every 85 ml of extract. The extract was then precipitated at -20 °C

for 30 h by addition of two volumes of cold anhydrous ethanol and four volumes of cold anhydrous ether. Precipitates were recovered by centrifugation at 6000 g for 30 min and dissolved in 3-4 ml of 1 M acetic acid per g of tissue. The acid-insoluble residue was discarded after centrifugation and the supernatant was dialyzed against phosphate-buffered saline, pH 7.2.

Western blot assays

Aliquots of dialyzed extracts containing 45 µg each of total soluble proteins from non-transgenic and transgenic plants were separated in a 16% Tris-tricine gel in non reducing conditions and transferred onto a nitrocellulose membrane. The membrane was probed with 2 µg.ml⁻¹ of monoclonal rabbit anti-hEGF antibody (R&D systems Inc., USA) followed by three washes with 50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween 20, and a second incubation step with alkaline phosphatase-linked goat anti-rabbit antibody diluted to 1:2000 (Jackson Ltd., UK). After a final wash, phosphatase activity was determined by a chromogenic reaction using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma Chemical Co., USA) as substrates.

ELISA assay

Quantification of hEGF was performed by ELISA using commercial anti-hEGF antibodies (R&D Systems Inc., USA). Microtiter plates were coated with 100 µl of monoclonal anti-hEGF (3 µg ml⁻¹) per well and incubated overnight at room temperature. After washing for three times with PBST (1X PBS, pH 7.4, 0.05% Tween 20) plates were blocked with 200 µl per well of PBS containing 1% BSA and 5% sucrose for 2 h at room temperature and washed again with PBST. Extracts of total soluble proteins or the dialyzed fractions were added to microtiter plates and incubated for 2 h at room temperature. For quantification, a standard reference curve was constructed using serial dilutions of commercial hEGF (Sigma Chemical Co., USA). After a new series of washes, wells were loaded with 100 µl (20 ng.ml⁻¹) of biotinylated anti-hEGF and incubated at room temperature for 2 h, washed again, and incubated for 20 min with 100 µl per well of a 1:2000 dilution of 125 µg ml⁻¹ streptavidin-peroxidase (Sigma Chemical Co., USA). Biotinylated antibody was detected with 100 µl per well of 3,3', 5,5' tetramethylbenzidine

(TMB) substrate solution (1 mg ml⁻¹ TMB, 2 µl H₂O₂ in 10 ml of phosphate-citrate buffer pH 5.5; Sigma Chemical Co., USA). Reactions were stopped with 50 µl of 1 M H₂SO₄ per well and quantified by OD measurements at 450 nm. Detection limit of the assay was 0.1 ng of hEGF per g of fresh tissue.

Radioreceptor assay

hEGF binding in dialyzed extract was analyzed by the radioreceptor assay described by Bussmann et al. (1996). Briefly, membranes from human placenta, previously stripped of endogenous growth factors by treatment with 50 mM glycine, 100 mM NaCl, pH 3.0, were incubated with 40,000 cpm of ¹²⁵I-hEGF tracer and various amounts of the acid-alcohol plant extract dissolved in TBSCA buffer (50 mM Tris, 2 mM MgCl₂, 1% BSA). The same growth factor used for iodination was employed to generate the standard curve. Incubation was stopped by placing the samples on ice and adding 2 ml TBSCA buffer (50 mM Tris, 10 mM MgCl₂, 0.1% BSA, 0.1% celite, pH 7.0). Membrane-bound growth factor was separated by centrifugation at 6,000 g for 30 min, the supernatant was discarded and the pellet counted in a LKB gamma spectrometer (LKB Instruments, Sweden). The sensitivity of the assay was 0.15 ng per tube. Mean intra-assay coefficient of variation was 9%. Nonlinear regression analysis and K_d determination was performed using GraphPad Prism version 3.02 for Windows (GraphPad Software, USA).

Cumulus cells expansion test

Bovine ovaries were collected from a slaughterhouse and transported to the laboratory in PBS buffer at 30 °C. Immature cumulus-oocytes complexes (COCs) were aspirated from 2 to 6 mm antral follicles with a 19-gauge needle attached to a 5 ml syringe. Only COCs with oocyte homogeneous cytoplasm and surrounded by compact cumulus were selected. Then, the oocytes were washed in maturation medium and randomly assigned to one of the different experimental groups. Maturation medium of each group was composed of Tissue Culture Medium 199 (TCM199) supplemented with Earle's salts (Gibco-BRL, USA), 2.2 g l⁻¹ bicarbonate, pH 7.4, 50 µg ml⁻¹ gentamicin and the extracts or factors to be tested. Plant extracts equivalent to 10 ng of hEGF (as determined by ELISA tests) or 10 ng of commercial hEGF were used in each group. All plant samples were normalized to

contain the same amount of total protein. COCs *in vitro* maturation was carried out in 50 µl droplets under mineral oil (5 COCs per droplet) in 35 mm Petri dishes (Nunc, Denmark) kept in a humidified atmosphere of 5% CO₂ in air at 39 °C for 24 h. After maturation, cumulus expansion was visually assessed under a stereomicroscope (Nikon, USA). The statistical differences in the proportion of COCs expansion were analyzed using Chi square test.

Results

Production and characterization of transgenic N. tabacum plants

Three genetic constructs were engineered by fusing the hEGF coding sequence to different regulatory and signal peptides sequences. Two of these constructs – referred to as ‘cytoplasmic’ versions – consisted of the hEGF coding sequence fused to either a simple (p35EGF) or long (p35(L)EGF) CaMV 35S promoter followed by the TMV translational enhancer (Figure 1A). A third construct (referred to as ‘apoplastic’ version), was created by adding the sequence encoding the AP24 signal peptide to the hEGF gene and fusing this chimera to the long CaMV promoter and the TMV enhancer (p35(L)APEGF; Figure 1B). After cleavage of the signal peptide, this version would allow expression of a mature hEGF molecule differing from the commercial molecule in that the first methionine residue is replaced by alanine. Transformation of these three constructs into *N. tabacum* was accomplished via *Agrobacterium* using leaf disc explants. Kanamycin-resistant plants were analyzed for the presence of the hEGF sequence by PCR amplification with specific primers (data not shown). As a result of this preliminary screening, 14, 17 and 20 plants transformed with p35EGF, p35(L)EGF and p35(L)APEGF, respectively, were isolated and subjected to further evaluation. A number of these plants were analyzed by Southern blot to assess transgene integration into the nuclear genome (Figure 2A). Transgene copy number varied between one to four copies in the different lines examined.

Expression levels of hEGF in transgenic tobacco leaves

hEGF expression levels in tobacco leaves were quantified by ELISA. As shown in Figure 3, in plants ex-

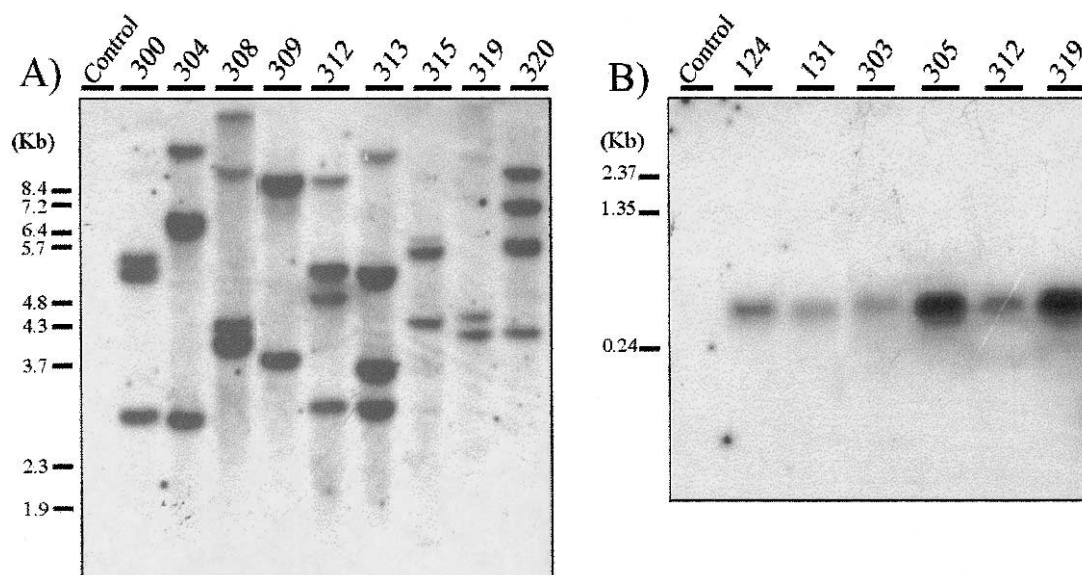


Figure 2. Southern and Northern blot analysis of selected transgenic lines. A) Southern blot of representative transgenic lines and untransformed control plants. Total genomic DNA (10 μ g per lane) were Hind III digested, separated on 0.8% agarose gel and blotted onto a nylon membrane. DNA molecular weight marker: λ BstEII digested (New England Biolabs, USA) B) Northern blot of selected transgenic lines and untransformed control plants. Total RNA (20 μ g per lane) was separated on 1.5% denaturing agarose gel and blotted onto a nylon membrane. RNA molecular weight marker: RNA ladder (Gibco BRL, USA). Specific DNA and mRNA sequences were detected by hybridization with 32 P-labeled hEGF cDNA probes. 124, 131: plants transformed with p35(L)EGF. 300, 303, 304, 305, 308, 309, 312, 313, 315, 319, 320: plants transformed with p35(L)APEGF. Control: untransformed plant.

pressing the cytoplasmic versions yields did not exceed 0.00001% of total soluble protein (equivalent to about 1 ng of hEGF per g of fresh leaf tissue). No significant differences in accumulation levels were found between plants in which the hEGF sequence was driven by a simple or a long version of the 35S promoter. On the other hand, plants transformed with the apoplastic version showed levels of up to 0.11% of total soluble protein. No correlation between transgene copy number and expression levels was found in the plants (Figure 2A, Figure 3). As shown in Figure 2B, transcription levels in some apoplastic transformed plants (303 and 312) were comparable to those from cytoplasmic versions (124 and 131), although EGF expression levels were quite different (Figure 3). This suggests that the low level of expression in the cytoplasmic versions is not due to reduced transcript levels. Plant 319, expressing the apoplastic hEGF version, was chosen for further analysis because it showed the highest accumulation level ($34.2 \pm 2.6 \mu$ g of hEGF per g of fresh leaf tissue).

hEGF expression in virus-infected plants

The hEGF sequence was introduced into two PVX-based vectors that allow its expression as both cytoplasmic and apoplastic forms (Figure 1C and D). Since the cloning site is located 39 bases downstream from the start codon of the PVX coat protein (CP) gene, the cytoplasmic version was fused in frame to the first 14 amino acids from the viral CP (pPVX-EGF; Figure 1C). Similarly, the apoplastic version comprised 14 amino acids from the PVX CP followed by 22 amino acids from the AP24 signal peptide (pPVXAPEGF; Figure 1D). After proteolytic processing, the apoplastic version allowed expression of a mature hEGF molecule differing from the commercial product in that the first methionine residue was replaced by an alanine.

Infectious RNA transcripts from pPVXEGF or pPVXAPEGF and pPVX3 were inoculated on expanding leaves of *N. benthamiana* plants. After one week, *N. benthamiana* plants infected with PVX or the two recombinant viruses showed symptoms characteristic of PVX infections (mild chlorotic lesions) in both inoculated and upper leaves. Presence of the virus in inoculated and upper leaves was confirmed

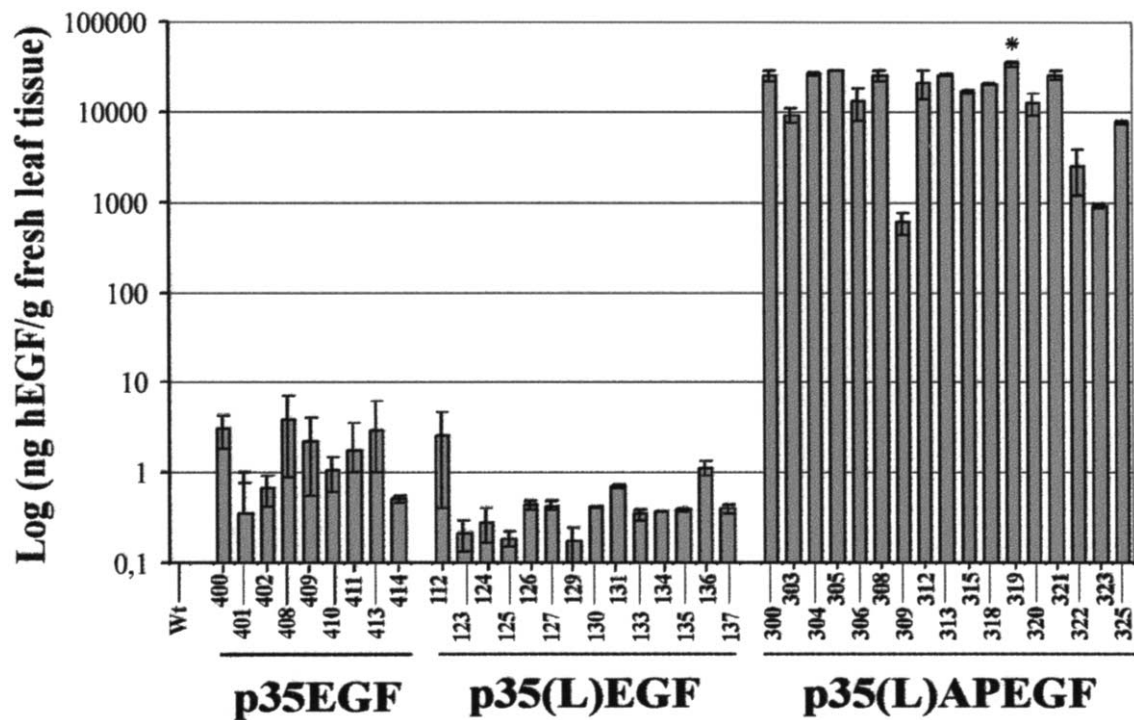


Figure 3. hEGF accumulation in transgenic plants. hEGF expressed in *N. tabacum* was quantified in leaf tissue extracts by ELISA. Data presented as the logarithm of ng hEGF per g of fresh tissue. Genetic constructs used for transformation are indicated for each group of plants. wt: non-transformed control plant. Values are the average of at least two independent measurements. Error bars represent standard deviations. hEGF accumulation in plant 319 is indicated by an asterisk.

by Western blot analysis with anti-CP antibodies (data not shown). Samples were collected at 18 and 30 days post-inoculation and analyzed for hEGF accumulation by ELISA. While hEGF was barely detectable in PVXEGF-infected plants, it accounted for up to 0.015% of total soluble protein in pPVXAPEGF-infected plants (Figure 4). No differences in hEGF accumulation were observed between 18 and 30 days post-inoculation. Extracts from *N. benthamiana* PVXAPEGF-infected leaves were used to infect *N. tabacum* var. Xanthi D8. As in *N. benthamiana*, symptoms were visible after a week in inoculated and upper leaves, confirming that the viral construct remained infective. hEGF expression levels were comparable in *N. tabacum* and *N. benthamiana* (Figure 4), but the number of leaf lesions was lower in the first.

Characterization of recombinant hEGF

Dialyzed protein extracts from the transgenic plant 319, expressing the apoplastic version p35S(L)APEGF, non-transgenic *N. tabacum* and PVX- and PVXAPEGF-infected *N. benthamiana*

plants, were separated in a tricine polyacrylamide gel and analyzed in Western immunoblots. As shown in Figure 5, a specific band co-migrating with commercial hEGF (6.2 kDa) was detected in plant 319 and the PVXAPEGF-infected plant and was absent in the respective controls. A smaller band, only detected in transgenic plant extracts, was putatively attributed to proteolytic degradation during the extraction procedure.

Dialyzed extracts from plant 319, non-transgenic *N. tabacum* and PVX- and PVXAPEGF-infected *N. benthamiana* plants were used to test hEGF binding and biological properties. A radioligand assay conducted with different concentrations of commercial hEGF and the dialyzed fraction of plant 319 showed saturation binding curves with K_d values of 0.7719 ± 0.0576 nM and 0.7020 ± 0.0295 nM respectively (Figure 6A). An independent assay using dialyzed extracts from PVXAPEGF infected plants and commercial hEGF showed saturation curves with K_d values of 0.6885 ± 0.0718 nM and 0.7340 ± 0.1272 nM respectively (Figure 6B). These suggest that the hEGF obtained from plant 319 or from plants infected

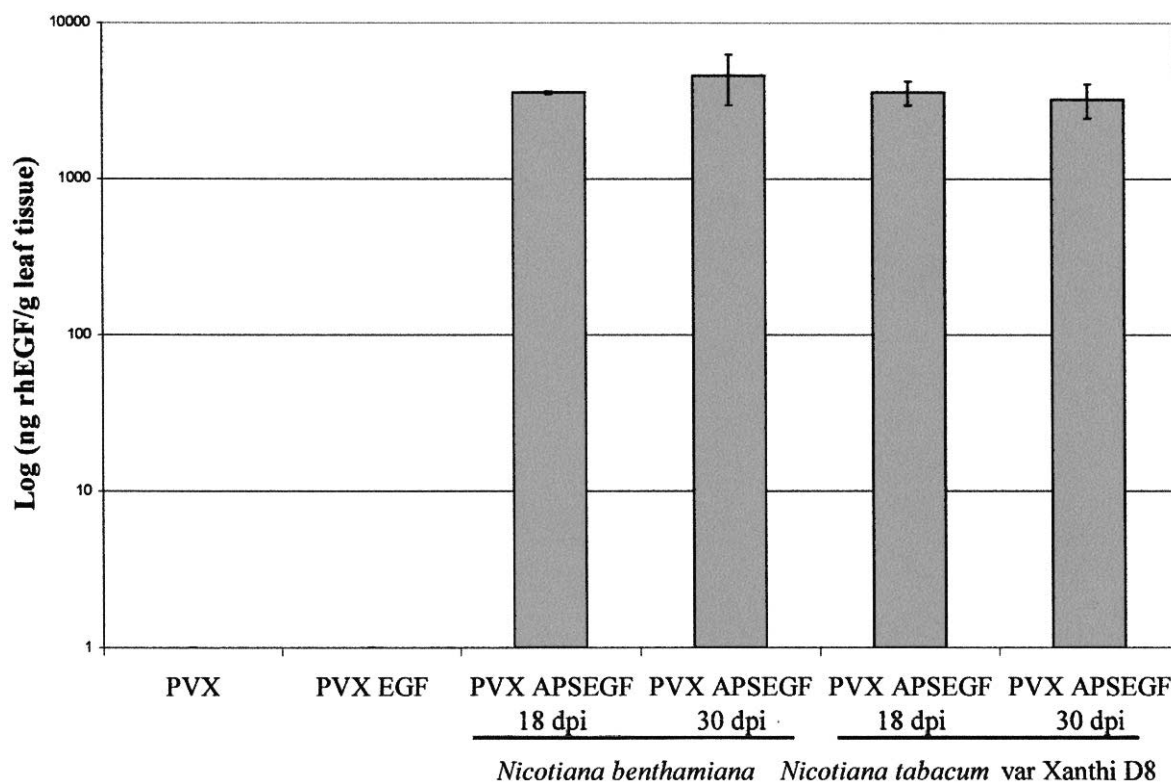


Figure 4. hEGF accumulation in *N. benthamiana*- and *N. tabacum*- infected plants. hEGF expressed plants infected with PVXEGF (cytoplasmic version) or PVXAPEGF (apoplastic version) was quantified in leaf tissue by ELISA assays. Viral vectors used for transformation are indicated for each group of plants. hEGF levels in PVXEGF-infected plants were under the detection limit of the assay. hEGF accumulation levels in PVXAPEGF-infected plants was measured at 18 and 30 days post infection. PVX: plants infected with empty PVX vector. Data are presented as the logarithm of ng hEGF per g of fresh tissue. Error bars represent standard deviations.

with the viral vector has a similar affinity for placental receptors than the commercial hEGF.

Further characterization of tobacco-expressed hEGF was obtained when extracts from *N. tabacum* 319, non-transgenic tobacco, and from PVX- or PVXAPEGF-infected *N. benthamiana* plants were assayed for cumulus cell expansion. Samples from plant 319 extracts and from PVXAPEGF-infected plants containing the equivalent of 10 ng of hEGF produced responses comparable to those obtained with 10 ng of commercial hEGF (Figure 7B, D and F). No expansion was observed in cumulus cells treated with control extracts (Figure 7A, C). Although a few COC treated with extracts of PVX-infected *N. benthamiana* seemed to show a slight cumulus expansion, the overall expansion rates was not statistically different from that observed in the control groups (Figure 7E).

Discussion

hEGF was expressed in tobacco plants using integrative and non-integrative expression systems. The firsts attempts to accumulate hEGF in the tobacco cell cytoplasm resulted in levels of about 0.00001% of total soluble protein. This percentage was similar for the constructs bearing a simple or a long version of the 35S promoter and was similar with previous results obtained by Higo et al. (1993). Since the hEGF level was not correlated with transgenic RNA accumulation (as visualized in Northern blot analysis), this low level was attributed to a high rate of proteolytic degradation. In order to improve these yields, the recombinant protein was targeted to the endoplasmic reticulum via the AP24 osmotin signal sequence. A similar approach was successfully implemented by others to accumulate different transgenic proteins in the apoplastic space (Fiedler and Conrad 1995; Firek et al. 1993; Herbers et al. 1995; Leite et al. 2000;

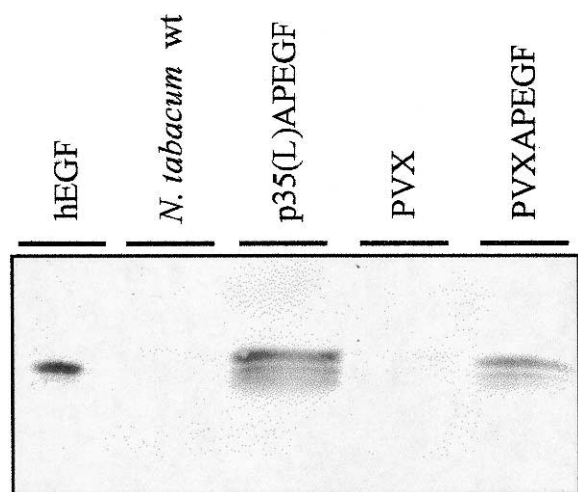


Figure 5. Western blot analysis of tobacco-expressed hEGF. Samples containing 45 μ g of total soluble protein from acid-alcohol extracts were loaded in each lane. Bands were detected with monoclonal anti-hEGF. *N. tabacum* wt: extract from non-transformed tobacco plant. p35(L)APEGF: extract from plant line 319. hEGF: commercial hEGF (20 ng). PVX and PVXAPEGF: extracts from *N. benthamiana* plants infected with PVX or PVXAPEGF, respectively.

McCormick et al. 1999). The apoplastic version was driven by the long 35S CaMV promoter and fused downstream of the Ω translational enhancer. As shown by ELISA quantification, this version allowed accumulation levels of up to 0.11% of total soluble protein, which represents a four-order increase com-

pared to the cytoplasmic versions. This effect must be mostly attributed to compartmentalization of the transgenic protein, since no significant differences were found between plants carrying constructs with different promoters or different transgene copy number. Additionally, in Northern blot analysis conducted in plants transformed with cytoplasmic or apoplastic versions, levels of hEGF mRNA were similar, suggesting that there is no limitation at the transcriptional level in the expression of cytoplasmic versions. This result paralleled those previously obtained by Higo et al. (1993). It has been established that protein transit through the endoplasmic reticulum is required for correct formation of disulfide bridges in several extracellular proteins. Since hEGF possesses three disulfide bridges, increased cytoplasmic degradation could result from improper folding of the recombinant molecule.

A purification protocol for extraction of transforming growth factors from mouse sub-maxillary glands (Roberts et al. 1980) was adapted to hEGF extraction from tobacco leaves. This semi-purified extract was suitable for biological *in vitro* assays. Two different tests were performed to characterize the behavior of tobacco-expressed hEGF. Firstly, recombinant hEGF showed a Kd value similar to that exhibited by commercial hEGF in saturation binding curves using placenta membrane receptors. Biological activity of the recombinant molecule was also confirmed by addi-

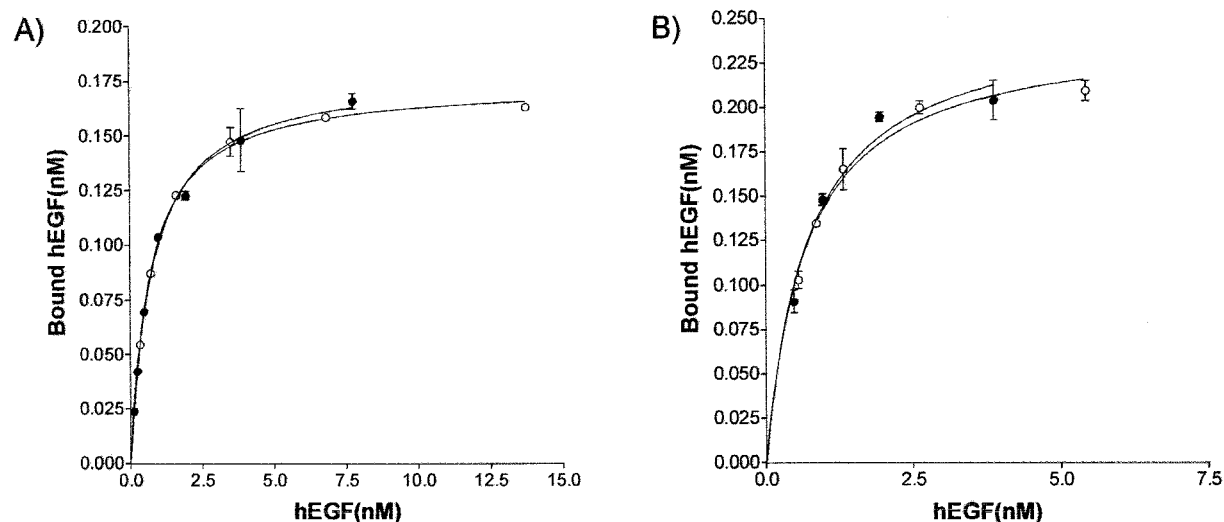


Figure 6. Saturation binding curves for commercial hEGF and acid-alcohol extracts from transgenic tobacco and extracts from *N. benthamiana* plants infected with PVXAPEGF. A) (○) Commercial hEGF and (●) acid-alcohol extract from plant 319. B) (○) Commercial hEGF and (●) acid-alcohol extract from PVXAPEGF-infected plants. Curves were calculated by non-linear regression using the GraphPad Prism version 3.02 program

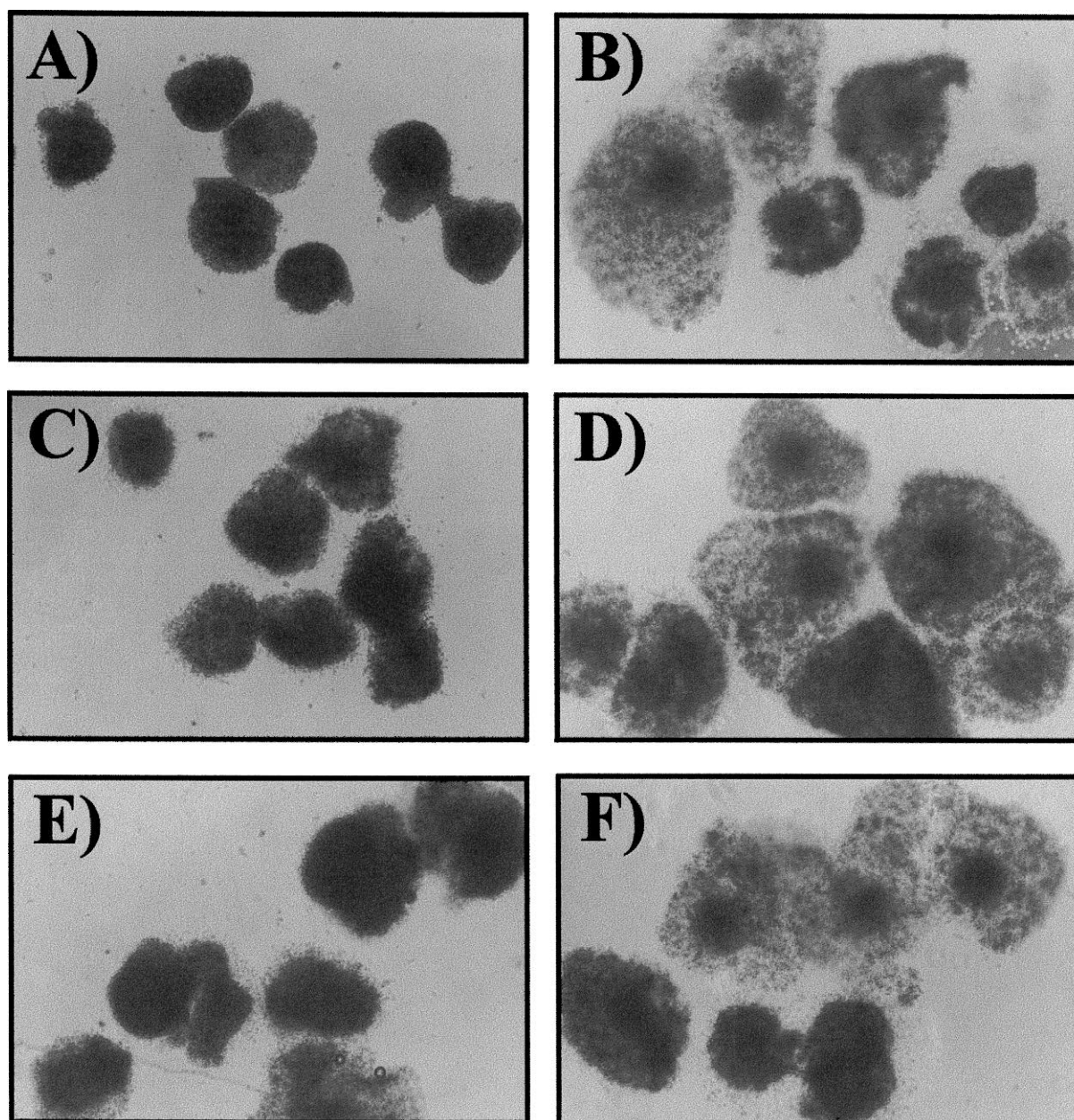


Figure 7. Cumulus cells expansion tests. A: no supplements added. B-F: the maturation medium was supplemented with, 10 ng of commercial hEGF (B), extract from *N. tabacum* non-transgenic plant (C), extract from plant 319 containing 10 ng of hEGF (D), extract from *N. benthamiana* plants infected with PVX (E), extract from *N. benthamiana* plants infected with PVXAPEGF (F).

tional *in vitro* assays using bovine cumulus cells. In this test, transgenic tobacco extracts showed a stimulatory effect on cumulus cell expansion comparable to that obtained with commercial hEGF

Use of viral vectors to test genetic constructs has the advantage of being less time-consuming than stable plant transformation. To compare hEGF accu-

mulation in plants infected with viral vectors with that obtained by transgene integration, a PVX-derived vector was designed in which the hEGF gene and a AP24-hEGF gene fusion were cloned under the control of a viral coat protein subgenomic promoter. No hEGF was detected in *N. benthamiana* plants infected with vector containing the cytoplasmic version

(PVXEGF). In contrast, plants infected with vector carrying the apoplastic version (PVXAPEGF) yielded up to 0.015% of total soluble protein. Leaves infected with this viral version were used to inoculate *N. tabacum* plants. Recombinant protein levels do not vary after this passage. Systemic infection could be established in both cases, thus showing that the recombinant virus remained infective.

Though use of viral vectors implies some advantages for the fast analysis of recombinant proteins, their use as a production system seems to be currently unfeasible for practical reasons. Thus, to develop a productive infection, plants must be inoculated at 4 weeks post-germination, when they are too small to be easily handled in the greenhouse or under field conditions. On the other hand, yields in virus-infected plants do not exceed in any case those obtained in transgenic plants. Therefore, a potential advantage of viral-based system – high expression levels – could not be exploited in this case.

There is no general rule for efficient production of recombinant proteins in plants and different approaches must be tested in each specific case. In this work, we demonstrate that biologically active hEGF can be expressed in transgenic tobacco plants and viral vector infected plants. From the different conditions tested here, it can be concluded that extracellular localization plays a critical role in protein yield increases in both transgenic and viral vector-infected plants. Tobacco is a non-edible plant that can easily be modified and cultivated at low production costs. Also, this crop generates a large volume of leaf biomass per hectare per year and, for this reason, it could be advantageous in obtaining high yields of the final product. Indirect estimations on hEGF concentration in tobacco leaves indicate protein levels that could be economically exploited at a commercial scale. Extraction of plant-expressed hEGF at a pilot scale is currently being undertaken to test this possibility.

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