

High-Level Transient Expression of ER-Targeted Human Interleukin 6 in *Nicotiana benthamiana*

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Abstract

Tobacco plants can be used to express recombinant proteins that cannot be produced in a soluble and active form using traditional platforms such as *Escherichia coli*. We therefore expressed the human glycoprotein interleukin 6 (IL6) in two commercial tobacco cultivars (*Nicotiana tabacum* cv. Virginia and cv. Geudertheimer) as well as the model host *N. benthamiana* to compare different transformation strategies (stable vs. transient expression) and subcellular targeting (apoplast, endoplasmic reticulum (ER) and vacuole). In T₀ transgenic plants, the highest expression levels were achieved by ER targeting but the overall yields of IL6 were still low in the leaves (0.005% TSP in the ER, 0.0008% in the vacuole and 0.0005% in the apoplast). The apoplast variant accumulated to similar levels in leaves and seeds, whereas the ER-targeted variant was 1.2-fold more abundant in seeds and the vacuolar variant was 6-fold more abundant in seeds. The yields improved in subsequent generations, with the best-performing T₂ plants producing the ER-targeted IL6 at 0.14% TSP in both leaves and seeds. Transient expression of ER-targeted IL6 in leaves using the MagniCON system resulted in yields of up to 7% TSP in *N. benthamiana*, but only 1% in *N. tabacum* cv. Virginia and 0.5% in cv. Geudertheimer. Although the commercial tobacco cultivars produced up to threefold more biomass than *N. benthamiana*, this was not enough to compensate for the lower overall yields. The recombinant IL6 produced by transient and stable expression in plants was biologically active and presented as two alternative bands matching the corresponding native protein.

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Introduction

Interleukin 6 (IL6) is a cytokine with several important physiological roles, including the regulation of immune responses, inflammation and hematopoiesis [1]. Excess IL6 is associated with various immune-mediated inflammatory diseases (IMIDs), including rheumatoid arthritis [2], atherosclerosis [3] and the neurodegenerative cascade leading to Alzheimer's disease [4]. IL6 is also involved in the progression of cancer [5,6] and diabetes [7]. Clinical studies have demonstrated that IL6 is a useful therapeutic target for certain IMIDs [8], but the development of novel drugs has been delayed by the limited availability of recombinant IL6.

Therapeutic proteins are generally manufactured in *Escherichia coli*, yeast or mammalian cells, despite the limited scalability of these platforms [9,10]. Complex proteins produced in *E. coli* tend to accumulate within inclusion bodies and these require labor-intensive resolubilization procedures that are generally avoided in commercial downstream processing [11,12]. IL6 also behaves in this manner when expressed in *E. coli* [13–17]. Aggregation can be avoided by using fusion tags, but tagging alters the protein structure and attracts a greater regulatory burden [12,18,19]. An alternative eukaryotic expression platform is therefore required to provide sufficient amounts of soluble and biologically-active IL6.

Leafy plants such as tobacco are potentially suitable because they have the ability to produce complex mammalian proteins [20], and can also be scaled up to into agricultural production systems with yields of up to 300 tons of biomass per hectare [21]. Tobacco is also advantageous because it is amenable to genetic engineering and numerous expression strategies are available [20]. Soluble IL6 has already been produced in the cytoplasm of tobacco cells, although the final yield was not determined [22]. However, recombinant proteins retained in the cytosol usually accumulate at low levels due to proteolytic degradation [23], and IL6 might be particularly vulnerable since it is also rapidly degraded in human plasma [24]. Targeting recombinant proteins to the secretory pathway can improve their stability and yield as well as permitting certain post-translational modifications that may improve biological activity, stability and pharmacokinetic properties [25,26].

Recombinant proteins can be expressed in tobacco leaves either transiently or by stable transformation. The latter approach is advantageous because predictable expression levels can be achieved in the offspring of a well-characterized transgenic event, which can give rise to a large population of homogeneous transgenic plants. Proteins can also be expressed in the seeds of transgenic plants, which is advantageous because seeds have low

levels of proteolytic activity [23,27] and contain fewer cross-purifying secondary metabolites that interfere with downstream processing [28]. One drawback in tobacco is that the seeds have a much lower biomass compared to leaves and seed production takes longer [21,27].

Even when using leaves, the transgenic approach is time consuming and expression levels are normally less than 5% TSP [29]. In contrast, the MagnICON transient expression system is based on a viral replicon [30]. Protein expression is therefore rapid, and recombinant protein concentrations of up to 80% TSP have been achieved in *Nicotiana benthamiana* [31]. This model host supports the efficient amplification of viral replicons due to the absence of functional RNA-dependent RNA polymerase 6 (RDR6), which is part of an endogenous anti-viral defense mechanism [32]. *N. benthamiana* has a lower biomass than *N. tabacum*, so the latter is more suitable for large-scale commercial production [33], but is also less susceptible to viral infection [32]. Numerous factors affect plant-virus interactions [34–36] therefore protein expression levels also differ significantly among different cultivars, as noted for human erythropoietin (EPO) and interleukin 10 (IL10) [33].

In order to develop an ideal plant-based expression platform for recombinant human IL6, we compared the transient expression and transgenic strategies in the commercial tobacco cultivars Geudertheimer and Virginia, which have not been used before for the production of recombinant proteins. We found that the MagnICON transient expression system produced much higher yields of IL6 than transgenic plants (approximately 100-fold higher) and that *N. benthamiana* was the most suitable host, achieving up to 7% TSP compared to 1% in cv. Virginia and 0.5% in cv. Geudertheimer, more than compensating for the reduced biomass accumulation in this species.

Materials and Methods

Construction of plant expression vectors

We designed a synthetic IL6 coding sequence (including the N-terminal signal peptide) based on the native human sequence (accession no. P05231-1) and codon optimized the sequence for expression in tobacco (www.kazusa.org). We also added three codons (GCT TCC TCC) after the ATG initiation codon to improve the efficiency of translation [37]. The gene constructs were synthesized by the DNA Cloning Service (Hamburg, Germany).

We used the binary transformation vector pLH9000 [38] (accession no. AF458478), which contains the neomycin phosphotransferase type II gene (*npII*) for selection [39] and the ColE1 and VS1 origins of replication for propagation in *E. coli* and *Agrobacterium tumefaciens*, respectively. We inserted a polylinker and expression cassette comprising the CaMV 35S promoter with double-enhancer [40], the tobacco mosaic virus (TMV) Ω -fragment [41] and the CaMV 35S terminator [40] between the SfiI sites in the vector, and then integrated the abovementioned synthetic gene constructs at the BamHI/EcoRI sites in the polylinker. This basic cassette resulted in protein targeting to the apoplast. For ER and vacuolar targeting, synthetic oligonucleotide sequences were designed based on the SEKDEL ER-retention signal [42], and the vacuole sorting determinant AFVY from Phaseolin [43]. The synthesized oligonucleotides (Invitrogen) were fused to the 3'-end of the coding region at the SacI/NruI sites. All vectors were verified by DNA sequencing (GATC Biotech AG, Konstanz/Germany).

The MagnICON transient expression vectors were provided by Nomad Bioscience (Halle/Saale, Germany). The cr-TMV/

TVCV-based vector pICH29912 was derived from the high-yielding vector pICH18711 [44] by replacing the green fluorescent protein (GFP) coding region with a BsaI cloning site. The *Potato virus X* (PVX) vectors pICH27727 and pICH31160 were identical, except that pICH27727 contained the GFP coding region whereas pICH31160 contained the *E. coli lacZ α* gene encoding β -galactosidase, flanked by BsaI restriction sites. The pICH27727 vector combined the 5'-PVX module of pICH21380 and the 3'-PVX module of pICH21470, as described elsewhere [45].

The cloning strategies were identical for the cr-TMV/TVCV (pICH29912) and PVX (pICH31160) plasmids. The coding region of IL6ER was amplified from pLH-IL6ER with additional BsaI restriction sites using either primer 29912-BsaI-NtIL6ER-fw or 31160-BsaI-NtIL6ER-fw in combination with NtIL6ER-BsaI-rv (Table 1). The PCR product was integrated into the BsaI sites of the target vectors, as described [46]. The vectors were verified by sequencing with primer pairs TMV-fw and TMV-rv or PVX-fw and PVX-rv (Table 1).

Vectors for stable transformation were transferred to *A. tumefaciens* strain C58C1, and those for transient expression were transferred to *A. tumefaciens* strain ICF320, which is a disarmed, auxotrophic derivative (Δ cysKa, Δ cysKb, Δ thiG) of strain C58 [47].

Stable transformation of tobacco plants

Wild type tobacco (*Nicotiana tabacum* cv. Geudertheimer) seeds were surface sterilized in saturated calcium hypochlorite solution and 0.1% Triton X-100 for 5 min. The seeds were rinsed with sterile distilled water several times to remove the detergent, and then germinated on Linsmaier and Skoog (LS) medium (4.4 g/l LS medium including vitamins; catalog no. L0230.0050; Duchefa, Belgium) supplemented with 30 g/l sucrose, 6.5 g/l plant agar (catalog no. P1001.1000; Duchefa, Belgium) and adjusted to pH 5.7. The plants were maintained at 24/22°C day/night temperature with a 16-h photoperiod.

Tobacco leaves approximately one month old were used for *Agrobacterium*-mediated transformation essentially as described [48] but optimized for the transformation of cultivar Geudertheimer by Tina Hausmann (personal communication). Regenerated shoots were selected on LS medium containing 100 μ g/ml kanamycin and 500 μ g/ml cefotaxim. Regenerated plants were transferred to peat soil in the greenhouse until they were mature. Transgene integration was confirmed by PCR (Table 1).

Transient expression in tobacco leaves

Transient expression in *N. benthamiana* and *N. tabacum* cv. Geudertheimer and cv. Virginia plants (6–9 weeks old) was carried out as described by Giritch *et al.* [45]. A bacterial smear was inoculated into 5 ml starter culture containing 50 μ g/ml rifampicin and 50 μ g/ml kanamycin, and was incubated overnight 28°C, 220 rpm. The overnight culture was sedimented (10 min, 4500 rpm, 4°C) and the pellet was resuspended in 50 ml infiltration buffer containing 10 mM MES (pH 5.5) and 10 mM MgCl₂ (1:100 dilution).

DNA analysis

The T-DNA cassette was detected by PCR analysis of crude leaf extracts prepared from 100 mg of leaf tissue homogenized under liquid nitrogen and resuspended in 200 μ l of extraction buffer (50 mM NaOH, 0.25% SDS). After boiling for 10 min and pelleting in a bench-top centrifuge, the supernatant was diluted 1:5 in distilled water. PCR was used to detect both the IL6 gene and the *npII* marker. After an initial denaturation step (95°C for 5 min) we carried out 39 amplification cycles (95°C for 1 min,

Table 1. Primers used in this investigation.

Primer	Sequence	Fragment length
NtIL6-fw	5'-CTGCTGCATTTCTCGCCAG-3'	
NtIL6-rv	5'-GTGTAGTCATGTCTTGCAACC-3'	486 bp
NtIL6ER-rv	5'-GATTAAGCTCATCCTTCTCAGAC-3'	568 bp
NtIL6Vac-rv	5'-CGCGATTAGTACACGAAAGCC-3'	568 bp
2npt-fw	5'-TCCGGCCGCTGGGTGGAGAG-3'	
2npt-rv	5'-CTGGCGGAGCCCTGATGCT-3'	469 bp
aadA-fw	5'-tgatttgctggttacggtga-3'	
aadA-rv	5'-atttgccgactaccttggtg-3'	646 bp
Actin-fw	5'-GCAACTGGGATGATATGGAGAA-3'	~1100 bp*
Actin-rv	5'-GTGCCTTTGCAATCCACATCTG-3'	~850 bp**
29912-BsaI-NtIL6ER-fw	5'-ttttGGTCTCACATGGCTTCTCCAATTCATTCTC-3'	693 bp
31160-BsaI-NtIL6ER-fw	5'-TTTTGGTCTCAAGGTATGGCTTCTCCAATTCATTCTC-3'	696 bp
NtIL6ER-BsaI-rv	5'-TTTTGGTCTCTAAGCTTAAAGCTCATCCTTCTCAGAC-3'	
TMV-fw	5'-GATCCGGACGTCGAAGGTTTCAAGG-3'	1409 bp ¹
TMV-rv	5'-CTTGACTCTAGCTAGAGCGGCCCTGG-3'	1304 bp ²
PVX-fw	5'-CCAGCTGCCATCATGCCAAAGAGGG-3'	1336 bp ³
PVX-rv	5'-GACTGATGGGCTGCCTGTATCGAGTGG-3'	1234 bp ⁴

¹pICH18711;²pICH29912-IL6ER;³pICH27727;⁴pICH31160-IL6ER;

*PCR on genomic DNA;

**PCR on cDNA according to Liu *et al.* [49].

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58°C for 1 min, 72°C for 2 min) and a final elongation step (72°C for 10 min). The primer pairs are listed in Table 1.

RNA analysis

Total RNA was isolated from 100 mg tobacco leaf tissue using Trizol reagent according to the manufacturer's instructions (Invitrogen). RNA integrity was assessed by visualizing the 28S and 18S rRNA bands under UV light in a denaturing 0.8% MOPS-agarose gel containing ethidium bromide. For reverse transcription (RT)-PCR analysis, total RNA samples were digested with DNase for 3 h and the removal of DNA was confirmed by PCR detection of the endogenous actin sequence, which generates different products from genomic DNA and cDNA templates [49]. We used the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's recommendations, with each reaction comprising 1 µg of DNase-treated RNA, 10 mM dNTP mix, 0.5 µg oligo(dT)-primer, the supplied 1× reaction buffer and 200 U reverse transcriptase. The reaction was incubated at 42°C for 60 min then stopped by heating to 70°C for 10 min. The PCR was carried out using the same parameters described for DNA amplification, but we used multiplex conditions including actin-specific primers so that transgene expression could be compared to the endogenous actin gene. The amplified PCR products were separated by 1.5% TAE-agarose gel electrophoresis in gels containing ethidium bromide for visualization.

Southern blot analysis

Genomic DNA was extracted from 3 g of leaf tissue using the cetyltrimethylammonium bromide (CTAB) method [50], and 50 µg of genomic DNA was digested overnight, separated by 1% TBE-agarose gel electrophoresis and transferred to a positively-charged nylon membrane (BioDyne[®] A 0.45 µm; Pall Life Science VWR; Darmstadt, Germany) by capillary blotting in 10× SSC. The DNA was fixed by UV cross-linking. The membranes were prehybridized in SDS phosphate buffer (7% SDS, 50 mM sodium phosphate (pH 7.0), 2% blocking reagent (Roche, Mannheim, Germany), 50% formamide, 5× SSC, 0.1% sodium lauroyl sarcosinate) at 42°C for 2 h, and probed with a DIG-labeled PCR fragment at 42°C overnight. Double-stranded DIG-labeled DNA probes were prepared by PCR using the DIG DNA Labeling Kit (Roche Mannheim, Germany), construct-specific primers (Table 1) and the corresponding binary vectors as the template. The probes were denatured by boiling for 10 min before hybridization. Membranes were washed twice at room temperature with 2× SSC, 0.1% SDS for 15 min, and then twice with 0.1× SSC, 0.1% SDS at 68°C for 20 min. Signal detection with an alkaline phosphatase-conjugated anti-DIG antibody was carried out using the DIG Nucleic acid Detection Kit (Roche, Mannheim, Germany). Blots were exposed on Kodak Biomax light film (VWR, Darmstadt, Germany).

Enzyme-linked immunosorbent assay (ELISA), western blot and protein purification

Leaf samples (150 mg) were homogenized in liquid nitrogen and resuspended in 250 μ l cold protein extraction buffer (250 mM sucrose, 50 mM Tris (pH 7.5), 1 mM EDTA, 2 mM PMFS, 0.1% Triton X-100). For seed samples, 150 mg homogenized seeds were resuspended in 500 μ l extraction buffer. Samples were centrifuged for 10 min in a cooled bench-top centrifuge and the protein concentration in the supernatant was measured according to the Bradford (1976) method using Pierce reagent with bovine serum albumin (BSA) as the standard (Thermo scientific, Bonn, Germany).

For the IL6 ELISA assay, a commercial Kit was used (Human IL-6 Ready-SET-Go ELISA; catalog no. 88-7066-86; eBioscience, Frankfurt, Germany). Recombinant IL6 was quantified according to the manufacturer's instructions. Briefly, 96-well plates were coated with a mouse anti-human IL6-specific antibody at a final concentration of 1 μ g/ml at 4°C overnight. Following five washes with PBS containing 0.05% Tween-20, the plates were incubated with 100 μ l diluted leaf extract at room temperature for 2 h. After another wash, the plates were incubated with the corresponding biotinylated detection antibody at room temperature for 1 h, washed again and incubated with streptavidin conjugated to horseradish peroxidase at room temperature for 30 min. Finally, the plate was incubated with tetramethylbenzidine (TMB) at room temperature for 15 min in the dark. The reaction was stopped with 250 mM sulfuric acid. Extinction was measured at 450 nm in a Synergy HT multidetection reader (Bio-Tek, Bad Friedrichshall, Germany).

For Western Blot analysis, total soluble protein was extracted from leaf and seed samples as described above. Samples 100 μ g of total soluble protein were resuspended in 1 \times sample buffer containing 10% glycerin, 150 mM Tris (pH 6.8), 3% SDS, 1% β -mercaptoethanol and 2.5% bromophenol blue. The samples were heated to 95°C for 5 min and separated under denaturing conditions by 15% SDS-PAGE and then electrophoretically transferred to a 0.45- μ m PVDF membrane (VWR; Darmstadt, Germany). The proteins were transferred at a constant 2 mA/cm² at room temperature for 2 h in a Bio-Rad Trans-Blot semi-dry transfer cell using 50 mM Tris, 40 mM glycine, 0.01% SDS and 20% methanol as the transfer buffer (pH of 8.5). The membrane was blocked with PBS containing 0.05% Tween-20 and 5% nonfat milk powder at room temperature overnight and then probed at room temperature for 2 h with a mouse monoclonal anti-human IL6 antibody (catalog no. MA1-35475; ABR BioReagent, USA) or a rabbit polyclonal anti-human-IL6 antibody (catalog no. AB1421; Millipore, Schwalbach, Germany), each at 1:1000 dilution. After three washes, the membrane was probed at room temperature for 2 h with a horseradish peroxidase (HRP)-conjugated secondary antibody, either goat anti-mouse (catalog no. 715-035-151; Dianova, Hamburg, Germany) or donkey anti-rabbit (catalog no. NA934V; GE Healthcare, Munich, Germany), each at 1:10000 dilution. The signal was detected by ECL chemiluminescence and the membrane was exposed to Kodak Biomax light X-ray film (VWR; Darmstadt, Germany) for 1 min before it was developed and fixed.

Analysis of IL6 biological activity

The biological activity of IL6 was evaluated in a hybridoma proliferation assay using the IL6-dependent murine hybridoma cell line B-9 [51]. The tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added after 3–4 days cultivation in the presence of IL6 and the formation of formazan was used to measure cell proliferation and thus the

concentration of active plant-derived IL6 compared to a commercial standard (catalog no. C-61601; PromoCell; Heidelberg, Germany).

Statistical methods

Statistical comparisons were carried out using the *F*-test (ANOVA including the Bonferroni post-hoc test) with $p \leq 0.05$ (two-sided) considered significant. The variability of different events and siblings was characterized by the relative coefficient of variation (CV, %) $V_r[\%] = \frac{1}{\sqrt{n}} \cdot \frac{s}{y} \cdot 100$. The experimental design was calculated with SPSS.

Results

Construction of expression vectors for stable transformation and transient expression

The IL6 expression vectors used for stable expression (Fig. 1a) and transient expression (Fig. 1b) were constructed by incorporating the codon-optimized human IL6 gene (Fig. 1c). Several different variants of each vector were created to achieve protein targeting to the apoplast, ER and vacuole (Fig. 1d). The sequences of all the transformation vectors were verified by DNA sequencing (data not shown).

Molecular analysis of transgenic tobacco plants

One of the objectives of this study was to investigate the suitability of the two commercial tobacco cultivars (*Nicotiana tabacum* cv. Geudertheimer and cv. Virginia) for the production of human IL6. Transgenic plants were selected on medium containing kanamycin and grown to maturity in the greenhouse. Total genomic DNA was prepared from the crude leaf extracts of putative T₀ transgenic plants and non-transgenic controls, and was screened for the presence of the IL6 sequence and the *nrpII* marker gene (data not shown) resulting in the expected amplification products.

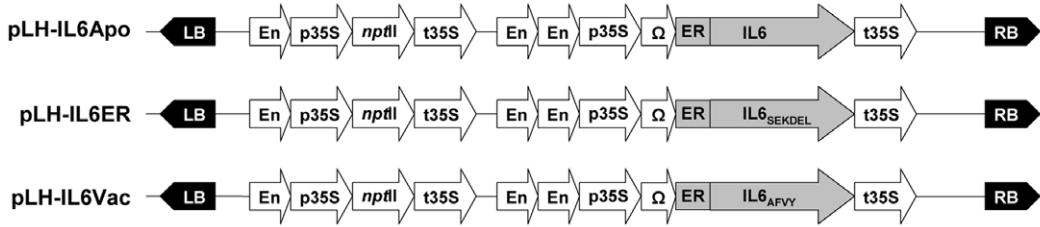
Transgene expression was analyzed by multiplex RT-PCR using the endogenous *actin* housekeeping gene as an internal control. Products of the expected size were detected in transgenic leaf tissue and the presence of the expected actin cDNA amplification product but the absence of the corresponding genomic product (1.1 kb) confirmed the absence of genomic DNA contamination (Fig. 2). There appeared to be no difference in mRNA expression levels between the three targeted variants of IL6.

IL6 accumulation in the subcellular compartments of leaves and seeds in T₀ plants

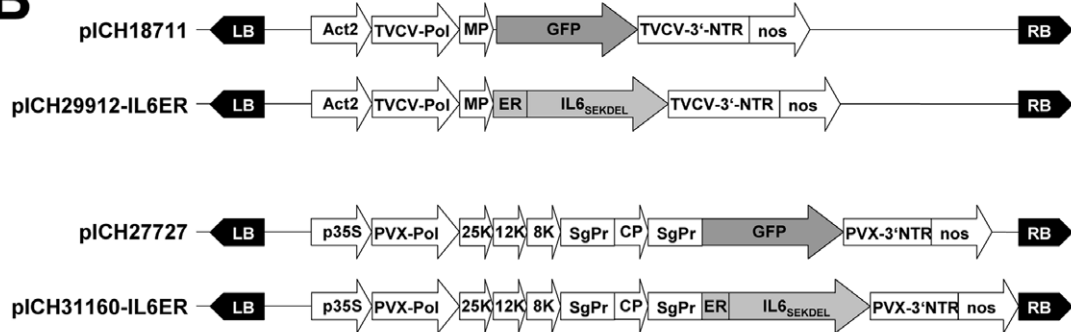
ELISAs were carried out on crude extracts from the uppermost fully-expanded leaves of 6-week-old plants to detect IL6 protein. As shown in Figure 3a, the ER-targeted variant was more abundant than the apoplast and vacuole versions, averaging 48.2 pg of IL6 per μ g TSP (Fig. 4a) and with a maximum of 73 pg IL6 per μ g TSP in the best-performing event IL6ER 66, corresponding to 5.9 μ g IL6 per g fresh leaf weight (Table 2). The average yields achieved using the other variants were approximately an order of magnitude lower, i.e. 5.79 pg IL6 per μ g TSP for IL6Apo and 5.21 pg IL6 per μ g TSP for IL6Vac (Fig. 4a). Moreover, the heterogeneity in the expression between the individuals was less pronounced as demonstrated by the CV values of 10.69 (IL6Apo) and 8.38 (IL6Vac), compared to the CV of 20.95 for the events, which carry the IL6ER construct (Fig. 3b).

We selected the ten best-performing T₀ plants for self-pollination, allowing the measurement of IL6 accumulation by

A



B



C

M	A	S	S	N	S	F	S	T	S	A	F	G	P	14
ATG	GCT	TCC	TCC	AAT	TCA	TTC	TCT	ACT	TCG	GCC	TTT	GGT	CCA	42
V	A	F	S	L	G	L	L	L	V	L	P	A	A	28
GTT	GCA	TTC	AGC	CTT	GGA	TTG	CTC	TTA	GTG	TTG	CCT	GCT	GCA	84
F	P	A	P	V	P	P	G	E	D	S	K	D	V	42
TTT	CCT	GCG	CCA	GTT	CCG	CCA	GGT	GAA	GAT	TCT	AAG	GAC	GTA	126
A	A	P	H	R	Q	P	L	T	S	S	E	R	I	56
GCT	GCG	CCA	CAC	AGA	CAA	CCA	TTA	ACC	TCT	AGT	GAG	AGA	ATT	168
D	K	Q	I	R	Y	I	L	D	G	I	S	A	L	70
GAT	AAG	CAG	ATA	CGA	TAC	ATT	CTC	GAT	GGA	ATC	TCT	GCT	CTG	210
R	K	E	T	C	N	K	S	N	M	C	E	S	S	84
AGG	AAG	GAG	ACT	TGT	AAC	AAA	TCT	AAT	ATG	TGC	GAA	TCA	TCT	252
K	E	A	L	A	E	N	N	L	N	L	P	K	M	98
AAG	GAA	GCT	CTA	GCC	GAG	AAC	AAT	CTT	AAT	CTG	CCT	AAG	ATG	294
A	E	K	D	G	C	F	Q	S	G	F	N	E	E	112
GCT	GAG	AAG	GAT	GGA	TGC	TTC	CAG	TCT	GGC	TTT	AAT	GAA	GAG	336
T	C	L	V	K	I	I	T	G	L	L	E	F	E	126
ACA	TGT	CTT	GTT	AAG	ATC	ATT	ACT	GGG	TTA	TTG	GAA	TTT	GAA	378
V	Y	L	E	Y	L	Q	N	R	F	E	S	S	E	140
GTG	TAT	CTC	GAG	TAT	CTT	CAG	AAC	AGG	TTT	GAA	TCA	TCT	GAG	420
E	Q	A	R	A	V	Q	M	S	T	K	V	L	I	154
GAA	CAA	GCT	CGT	GCT	GTT	CAG	ATG	AGT	ACT	AAA	GTC	CTT	ATA	462
Q	F	L	Q	K	K	A	K	N	L	D	A	I	T	168
CAA	TTC	CTT	CAG	AAG	AAG	GCT	AAG	AAT	CTT	GAT	GCC	ATT	ACG	504
T	P	D	P	T	T	N	A	S	L	L	T	K	L	182
ACT	CCT	GAT	CCA	ACA	ACC	AAC	GCC	TCT	CTT	CTT	ACT	AAG	TTG	546
Q	A	Q	N	Q	W	L	Q	D	M	T	T	H	L	196
CAA	GCT	CAG	AAT	CAA	TGG	TTG	CAA	GAC	ATG	ACT	ACA	CAT	CTT	588
I	L	R	S	F	K	E	F	L	Q	S	S	L	R	210
ATT	CTT	AGA	AGC	TTC	AAG	GAA	TTT	CTT	CAA	TCT	AGT	CTT	AGA	630
A	L	R	Q	M										215
GCT	CTT	AGG	CAA	ATG										645

D

Apoplasm:		*		218				
		TAA		648				
ER-Retention Signal:	S	E	K	D	E	L	*	224
	TCT	GAG	AAG	GAT	GAG	CTT	TAA	666
Vacuole Sorting Signal:	A	F	V	Y	*			222
	GCT	TTC	GTG	TAC	TAA			660

Figure 1. IL6 sequence and expression constructs. (A) Schematic representations of the T-DNA constructs used to express human IL6 in tobacco and target the recombinant protein to different subcellular compartments. LB: left border; RB: right border; En: -340 bp to -91 bp CaMV 35S enhancer; p35S: -90 to -1 bp CaMV 35S core promoter; t35S: CaMV 35S terminator; Ω : 5' *Tobacco mosaic virus* (TMV) untranslated region; *nptII*: nopaline phosphotransferase gene; IL6: synthetic human IL6 gene, including the native ER-targeting signal; SEKDEL: ER retention motif; AFVY: vacuole-targeting peptide from common bean phaseolin protein. (B) Binary 'MagniCON' vectors used for transient expression. TVCV-Pol: RNA-dependent RNA polymerase from *Turnip vein clearing virus* (TVCV); MP: TMV movement protein; TVCV-3'-NTR: TVCV 3' untranslated region; nos: *A. tumefaciens* nopaline synthetase gene terminator; PVX-Pol: polymerase/replicase from *Potato virus X* (PVX); 25K, 12K, 8K: PVX triple gene block; SgPr: subgenomic promoter; CP: PVX coat protein; 3'CP: 3' fragment of CP coding sequence; PVX-3'-NTR: PVX 3' untranslated region. (C) Protein and nucleotide sequence of human IL6 codon-optimized for tobacco. (D) Compartment specific C-terminal variants of IL6.
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ELISA in 150-mg batches of seeds. The average yield of IL6ER was 59.6 pg IL6 per μ g TSP, approximately 20% higher than in leaves (Fig. 4a), and the best-performing line IL6ER 66 yielded up to 84.3 pg IL6 per μ g TSP, corresponding to 21.1 μ g/g fresh seed weight (Table 2). The average yield achieved using the IL6Apo variant was similar to that achieved in leaves, and was again approximately an order of magnitude lower than the ER variant (5.95 pg IL6 per μ g TSP; Fig. 4a). Remarkably, the IL6Vac plants accumulated almost six times more IL6 in the seeds than in the leaves (27.82 pg IL6 per μ g TSP; Fig. 4a). Using the fresh weight of leaves or seeds as the parameter, the accumulation of IL6 in seeds was nearly four-fold greater in IL6ER and IL6Apo plants, and up to 16-fold greater in IL6Vac plants (Fig. 4b). There was no significant difference in the level of IL6 in dry seeds immediately after harvest and after three months in storage (Fig. 4c).

IL6 yields in T₁ and T₂ progeny

The three T₀ transformants with the highest IL6 yields (IL6ER lines 31, 66 and 79; Fig. 3), were self-pollinated to produce transgenic plants up to the T₂ generation. The seedlings were selected for kanamycin resistance prior to ELISA experiments.

In IL6ER lines 31 and 66, all T₁ descendants contained more IL6 in the leaf tissue than their T₀ parents, with a five-fold increase in line 31 and a ten-fold increase in line 66 (Fig. 5a). The best-performing T₁ plant (IL6ER 66-22) produced up to 730.9 pg IL6 per μ g TSP in its leaves, corresponding to 58.5 μ g/g fresh leaf weight (Table 2), and up to 1094.3 pg IL6 per μ g TSP in its seeds, corresponding to 273.6 μ g/g fresh weight. The T₁ generation of IL6ER line 79 did not show any improvement compared to the T₀ parent. IL6 expression levels were relatively homogenous between T₁ siblings, indicated by CVs of 7.27, 9.02 and 8.27 for the T₁ generation of lines IL6ER 31, 66 and 79, respectively (Fig. 5b).

IL6ER 66-22 was self-pollinated to generate T₂ progeny, only four of which showed a further improvements in IL6 yields (Fig. 5c). Moreover, the CV of 6.35 of T₂ generation was lower compared to that of T₁ generation (CV of 9.02) of the line IL6ER 66. The best-performing T₂ plant (IL6ER 66-22-17) produced up to 1397.6 pg IL6 per μ g TSP in its leaves, corresponding to 111.8 μ g/g fresh leaf weight, which is a 1.3-fold increase over the T₁ generation (Table 2). The same plant also produced up to 1212.3 pg IL6 per μ g TSP in its seeds, corresponding to 303.1 μ g/g fresh seed weight. Despite these isolated exceptions, most of the T₂ plants accumulated less IL6 than the T₁ event from which they were derived (Fig. 5c).

Correlation between IL6 expression and transgene copy number

Southern blot analysis of the transgene copy number, using a probe that targets the coding region of mature IL6 (Fig. 6a), revealed a complex integration pattern in line IL6ER 31, with four insertions that segregated to produce T₁ individuals with 1, 2 and 4 transgene loci (Fig. 6b). All the better-performing plants contained band 4 (31-1, 31-11 and 31-13), and the best-performing plant (31-1) contained bands 1 and 4 alone. Plants with all four signals (31-11 and 31-13) had a slightly lower expression than 31-1 and the worst-performing plants contained only bands 1 and 3 (31-10) or band 3 (31-14).

Lines IL6ER 66 (Fig. 6c) and IL6ER 79 (Fig. 6d) had a single integration locus. Despite carrying the same locus, T₁ plants from line IL6ER 66 were characterized by heterogeneous expression levels (Fig. 5b) ranging from 203.5 (line 66-3) to 730.9 (line 66-22) pg IL6 per μ g TSP. In contrast, the T₁ progeny from line IL6ER 79 showed relatively homogeneous expression levels comparable to the T₀ parent (Fig. 5a).

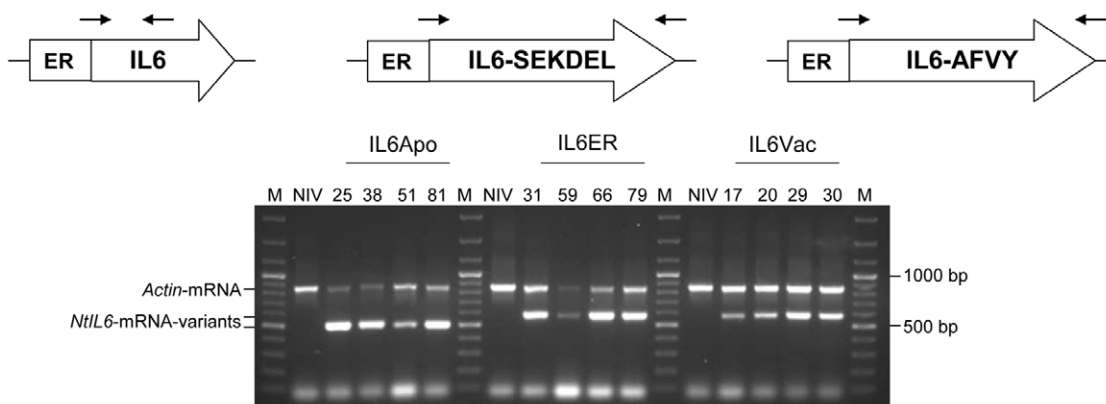


Figure 2. Gene expression of the IL6 constructs. Detection of IL6 mRNA variants in representative transgenic tobacco lines by RT-PCR. Total RNA was extracted from transgenic leaves to demonstrate the presence of *IL6Apo*, *IL6ER* and *IL6Vac* mRNAs using construct-specific primer (Table 1). Numbered lanes represent transgenic lines, NIV represents untransformed control plants. The *actin* sequence was amplified as an internal control, with anticipated products of 850 bp for cDNA and 1100 bp for genomic DNA. M indicates molecular size markers (GeneRuler DNA Ladder Mix; Fermentas, St. Leon-Rot, Germany).
doi:10.1371/journal.pone.0048938.g002

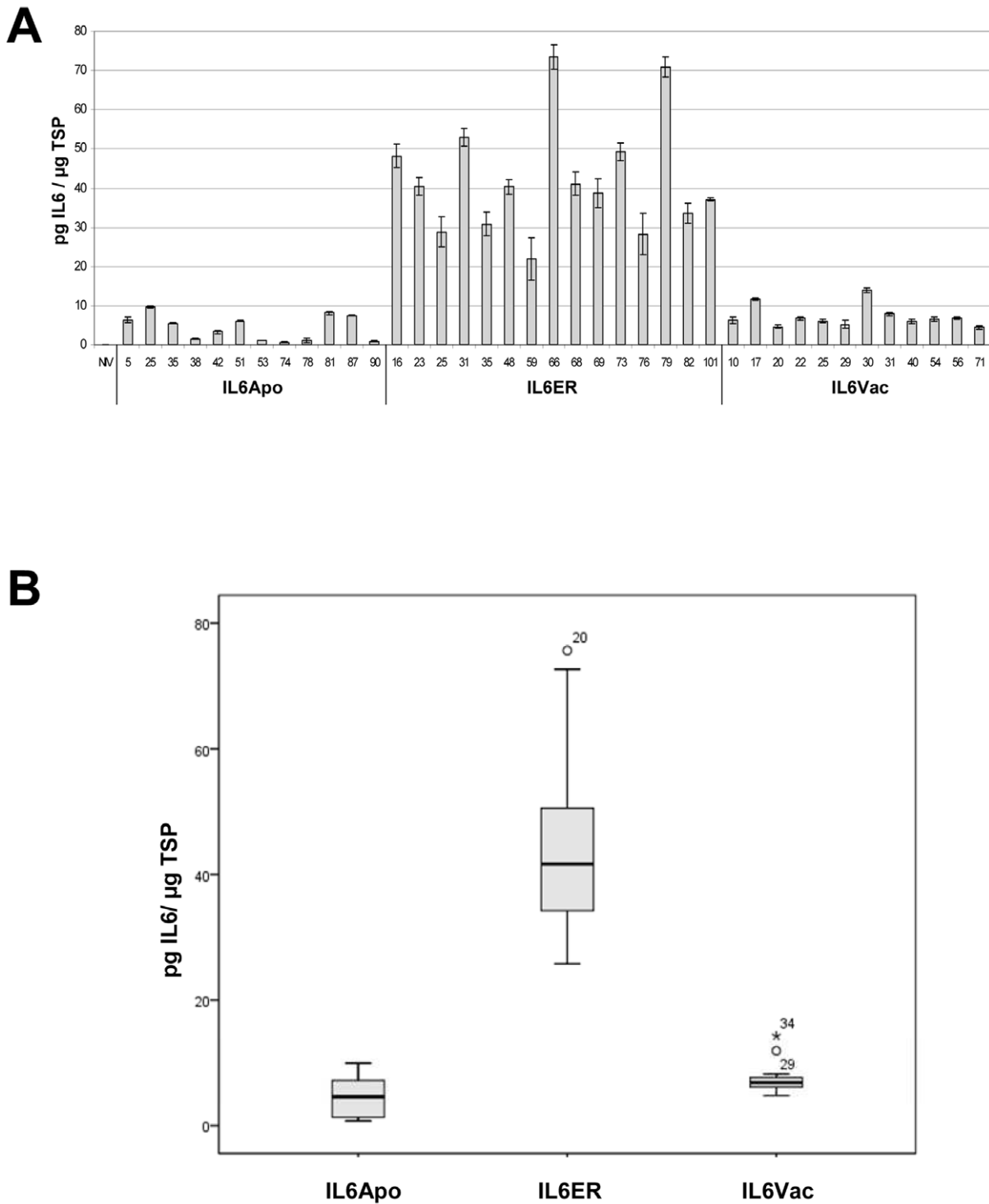


Figure 3. IL6 accumulation levels in T₀ transformants. (A) Protein expression levels in transgenic leaves expressing different IL6 variants, determined by ≥ 2 independent ELISAs. Numbered lanes represent transgenic lines, NIV represents untransformed control plants. The selected transgenic tobacco lines were derived from three independent transformation events. (B) Box plot representation of IL6 accumulation in the leaves of T₀ transgenic plants following ANOVA (including the Bonferroni post-hoc test). doi:10.1371/journal.pone.0048938.g003

MagnICON transient expression in commercial tobacco cultivars

Under our cultivation conditions, the two commercial tobacco cultivars produced up to three times as much biomass as *N.*

benthamiana (Fig. 7a). On average, cv. Geudertheimer produced 1.8-fold more and cv. Virginia produced 2.4-fold more harvestable biomass in 8–9-week-old plants.

The suitability of the three host plants was established by transient expression using control cr-TMV/TVCV and PVX

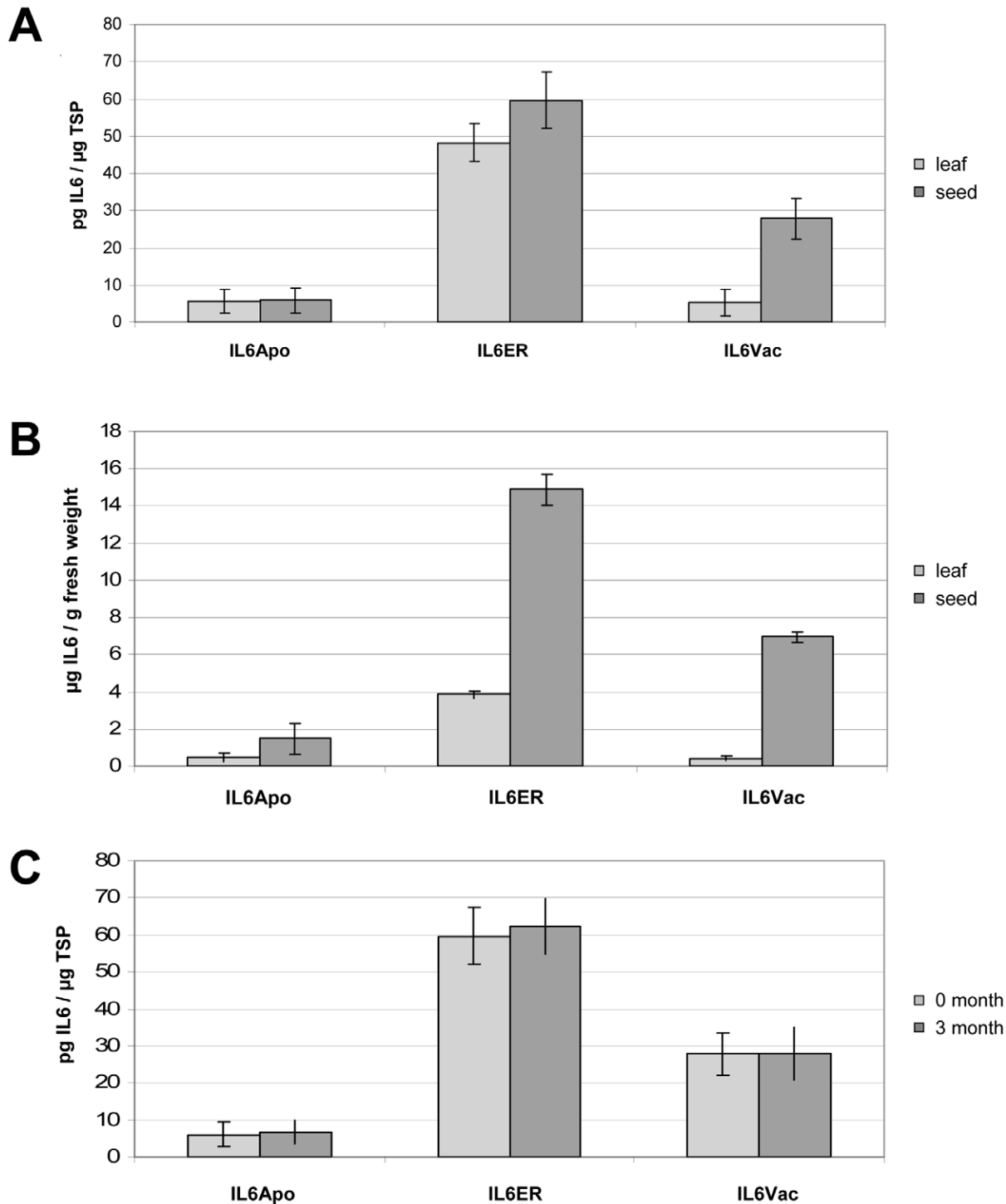


Figure 4. IL6 yield in leaves and seeds of T_0 transformants. Average protein expression levels in the leaves and seeds of ten T_0 transgenic tobacco plants expressing different IL6 variants, using either TSP (A) or fresh weight (B) as the reference parameter, determined by ≥ 2 independent ELISAs. (C) Average IL6/TSP content in the seeds of ten transgenic T_0 tobacco plants expressing different IL6 variants, measured after harvest (0 month) and after storage for 3 month at ambient conditions, determined by ≥ 2 independent ELISAs. doi:10.1371/journal.pone.0048938.g004

vectors carrying the GFP coding sequence (pICH18711 and pICH27727, respectively), followed by fluorescence analysis 10 days post inoculation (dpi). We tested two virus platforms because cr-TMV/TVCV can spread only by cell-to-cell movement in *N. benthamiana*, whereas PVX can also spread systemically [35,36,52].

The resulting fluorescence assays (Fig. 7b) and the analysis of Coomassie-stained SDS-polyacrylamide gels (data not shown), showed that the highest yields were achieved in *N. benthamiana*, with the Virginia cultivar approximately one order of magnitude lower and the Geudertheimer cultivar approximately two orders of

Table 2. Expression levels in leaves and seeds of the best-performing line IL6ER 66 and its progeny, using total soluble protein or fresh weight as the reference parameter.

construct	IL6ER					
	T ₀		T ₁		T ₂	
generation	66		66-22		66-22-17	
individual	66		66-22		66-22-17	
tissue	leaf	seed	leaf	seed	leaf	seed
IL6/TSP	73.5	84.3	730.9	1094.3	1397.6	1212.3
[pg/μg]						
IL6/fresh weight	5.9	21.1	58.5	273.6	111.8	303.1
[μg/g]						

doi:10.1371/journal.pone.0048938.t002

magnitude lower. These host-specific differences in the accumulation of GFP were observed both for the TMV and PVX vectors, although the PVX vectors produced less fluorescence (Fig. 7b). As expected, the PVX vectors spread both by cell-to-cell and systemic movement in *N. benthamiana*, but only by cell-to-cell movement in the commercial tobacco varieties (Fig. 7b).

Transient expression of IL6ER using the MagnICON system

Having demonstrated the transient expression of GFP in the commercial tobacco cultivars, we agroinfiltrated the leaves of both tobacco cultivars and *N. benthamiana* (by syringe) with the IL6 expression constructs. We expressed IL6 both in cr-TMC/TVCV vector pICH29912 which promotes high expression levels and in the PVX vector pICH31160 for reduced expression levels. In some instances, when high amounts of the target protein are accompanied by cytotoxic effects, a strong replicon leads to reduced expression levels of the target protein and the PVX is preferable (Dr. Anatoli Giritch, personal communication). Over-expression of IL6 did not lead to cytotoxic effects and highest yield - measured by ELISA - was achieved by the cr-TMV/TVCV vector (Fig. 8). IL6 was generally expressed at lower levels than GFP (data not shown) but the differences in performance among the three hosts showed a similar trend to GFP expression, with *N. benthamiana* giving the best performance (up to 7% TSP with the TMV vector), followed by cv. Virginia with up to 1% TSP and cv. Geudertheimer with up to 0.5% TSP (Fig. 8 and Table 3). Although the two commercial cultivars produced more biomass than *N. benthamiana* over the same time period (Fig. 7a) this was more than offset by the superior IL6 yields achieved in *N. benthamiana*.

Structural and functional analysis of recombinant plant-derived IL6

Total soluble protein samples (10–100 μg) were separated by 15% SDS-PAGE and characterized by western blot using antibodies raised against human IL6. Two distinct bands (~23 and ~28 kDa) were observed in samples from transgenic leaves and seeds (Fig. 9a) and agroinfiltrated leaves (Fig. 9b) with differences in expression levels consistent with the ELISA data (Fig. 8 and Table 3). The *E. coli* derived IL6 was found at ~21 kDa, which correspond to the molecular size of the mere amino acid sequence of mature IL6.

Crude leaf extracts from T₀ plants were used for IL6 bioassays based on *in vitro* cell proliferation in a murine cell line dependent

on IL6 (Fig. 9c). The recombinant IL6 stimulated cell proliferation in a dose-dependent manner, with a similar specific activity to a commercial standard derived from *E. coli*. We calculated an EC₅₀-value of 24 pg/ml for the IL6ER extract, whereas crude extracts from untransformed tobacco controls did not induce cell growth (data not shown).

Discussion

Tobacco has a long and successful history in molecular farming and is one of the key host species considered suitable for commercial exploitation [20,53]. We therefore investigated whether tobacco is suitable for the production of human IL6 by comparing transient and stable expression using a variety of targeting strategies.

ER-targeted IL6 accumulates to high levels in the leaves and seeds of transgenic plants

We compared three targeting strategies, i.e. directing the protein to the ER, the apoplast and the vacuole. The highest yields in the transgenic plants were achieved by ER targeting, in agreement with previous studies which have shown that ER retention improves the accumulation of most recombinant proteins in leaves and seeds [54,55]. This may reflect a prolonged interaction with ER-resident chaperones to promote correct folding [23] and/or the limited proteolytic activity in the ER compartment [26]. The latter is particularly relevant in the case of IL6 because it is vulnerable to proteases in human plasma [24]. IL6 may therefore be unstable in the apoplast due to the abundance of proteases [23].

Targeting IL6 to the protein storage vacuoles (PSVs) was expected to promote accumulation because this compartment also lacks significant proteolytic activity, in contrast to lytic vacuoles [56]. The PSV-sorting determinant we used has already been shown to promote the accumulation of human complement component C5a in tobacco leaves and seeds [57]. Therefore, the low yields of vacuole-targeted IL6 we achieved were unexpected. IL6 and C5a may be affected differently by certain vacuole-specific factors, a phenomenon already demonstrated in maize seeds with vacuole-targeted E1 cellulase and cellobiohydrolase I [58]. The E1 cellulase accumulated at high levels as expected but the cellobiohydrolase I was not detectable and the authors speculated that the proteins were sensitive to different compartment-specific proteases or protease inhibitors [58]. However, Hühns *et al.* [59] found that targeting signals do not always direct chimeric proteins to the anticipated location. The enzyme cyanophycin synthetase was combined with different chloroplast targeting signals, but only the PsbY signal functioned correctly [59] whereas in the other cases the recombinant protein was found in the cytoplasm. The choice between PSVs or lytic vacuoles as a targeting destination may therefore depend on the specific combination of sorting determinant and recombinant protein, and it is possible that the combination of our PSV import signal combined with IL6 resulted in targeting to lytic vacuoles followed by rapid degradation.

The ER and apoplast versions of recombinant IL6 accumulated to approximately the same levels (percentage TSP) in leaves and seeds, with no difference for the apoplast variant and only a 1.2-fold difference for the ER variant. Low levels in the apoplast were anticipated because this compartment contains abundant proteases in both leaves and seeds [23,27]. The marginally higher yield of the ER-targeted IL6 in seeds was unexpected, given that previous studies using the same promoter and the recombinant,

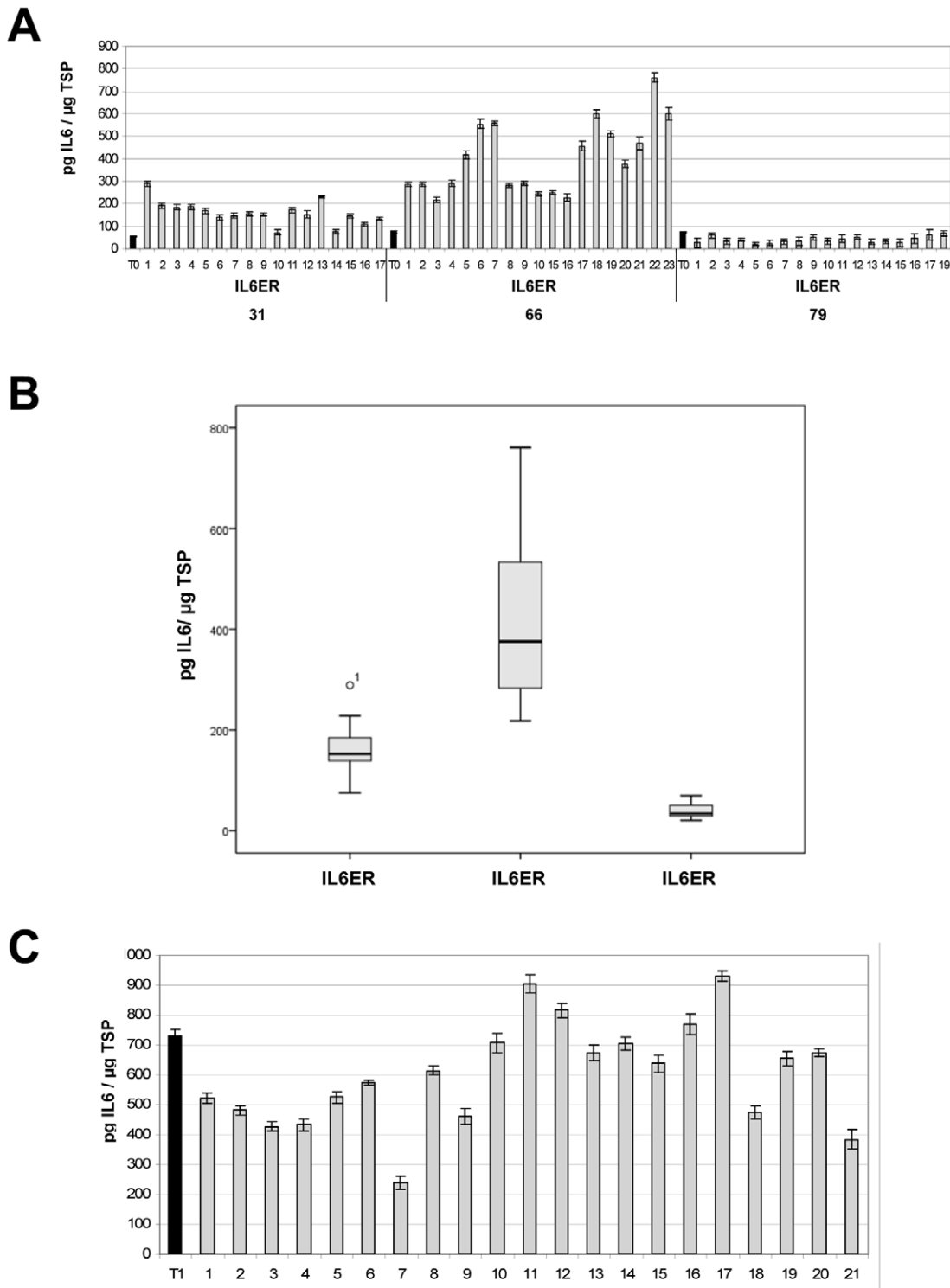


Figure 5. IL6 accumulation levels in T₁ transformants. (A) Protein expression levels in the T₁ generation of three independent transgenic tobacco lines expressing ER-targeted IL6 (31, 66 and 79), determined by ≥ 2 independent ELISAs. Numbers represent individual T₁ plants and T₀=the original transformant used for self-pollination. (B) Box plot representation of IL6 accumulation in leaves of T₁ progeny following ANOVA (including the Bonferroni post-hoc test). (C) Protein expression levels in the T₂ generation of transgenic tobacco line IL6ER 66-22, determined by ELISA. Numbers represent individual T₂ plants and T₁=original T₁ individual used for self-pollination. doi:10.1371/journal.pone.0048938.g005

ER-targeted antibody 14D9 recorded a more than 10-fold difference between the two tissues, with 5% TSP achieved in leaves and only 0.4% TSP in seeds [60]. The CaMV 35S

promoter is thought to be less active in seeds than in leaves [27], explaining the difference observed by Petrucelli *et al.* [60]. Our results may indicate that the lower promoter activity in seeds is

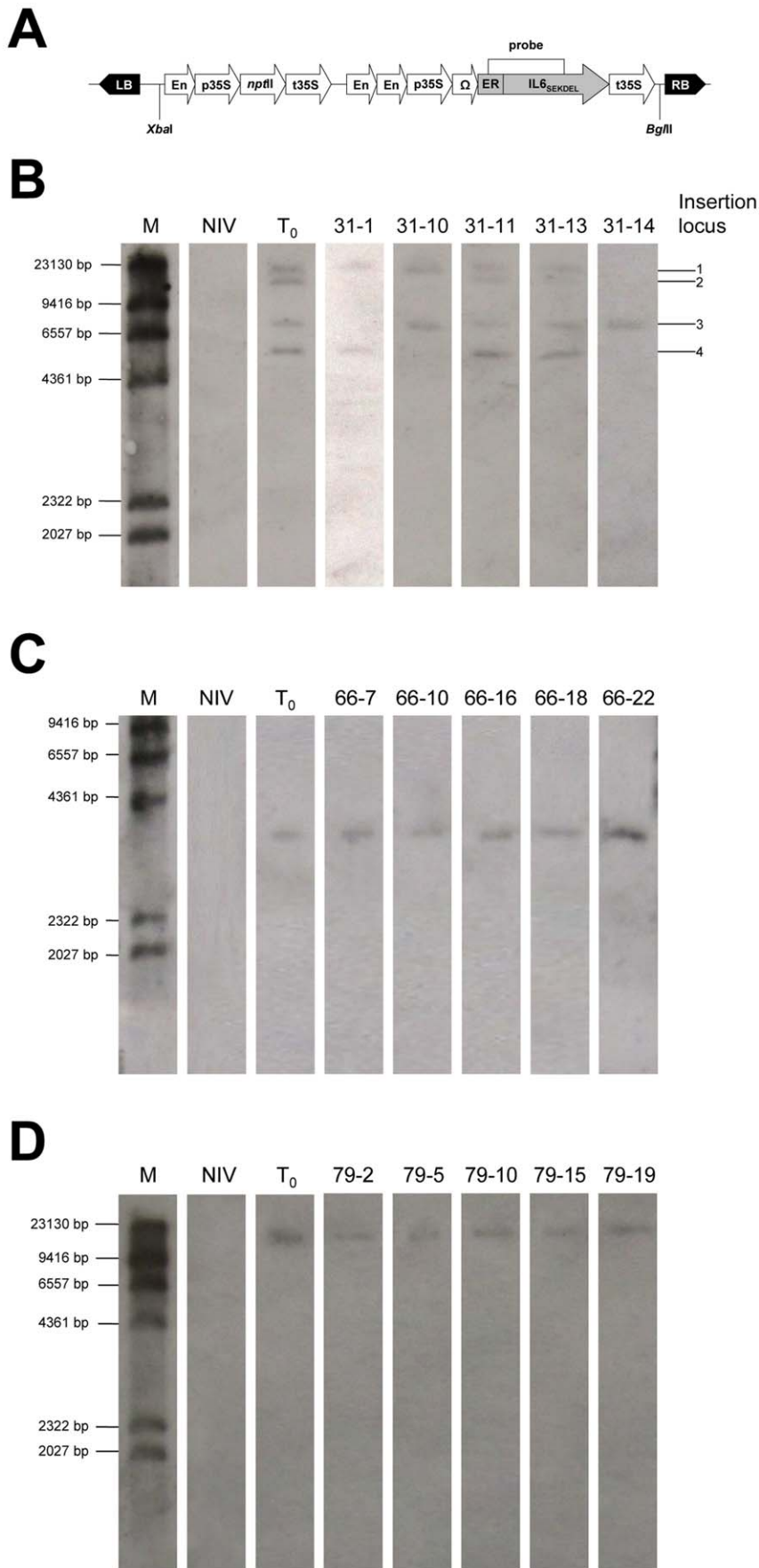


Figure 6. Southern blot analysis of T₀ parents and selected T₁ progeny from the transgenic IL6ER lines. Chromosomal DNA was digested with BglII, cutting cut once in the expression cassette. M: DIG-labeled DNA Molecular Weight Marker II (Roche); NIV: Untransformed negative control; Numbered lanes represent T₁ individuals. (A) Schematic representation of the integrated T-DNA structure and the probe position. (B) Southern blot of the line IL6ER 31. (C) Southern blot of the line IL6ER 66. (D) Southern blot of the line IL6ER 79. doi:10.1371/journal.pone.0048938.g006

compensated by the lower endogenous proteolytic activity [27] resulting in greater IL6 accumulation in the seeds.

The vacuolar version IL6 was almost six times more abundant in seeds than in leaves, based on percentage TSP (Fig. 4a). This may reflect the greater number of PSVs in seeds compared to leaves, and/or the species-dependent and tissue-specific functionality of sorting determinants [61]. Some proteins equipped with sorting determinants are correctly targeted in some tissues and incorrectly targeted in others of the same plant [56]. For example, vacuolar targeting of the DP1B protein, expressed in *Arabidopsis thaliana*, did show a tissue-specific impact on the stability and yield of recombinant protein [62]. IL6 may therefore be targeted to lytic vacuoles in leaves and to PSVs in seeds. Even with correct

targeting, the vacuolar sorting of IL6 produced lower yields than ER sorting, which can be regarded as the most suitable targeting strategy.

The transient expression of IL6 is more efficient in *N. benthamiana* than in commercial tobacco cultivars

Transient expression systems are more likely to be used for the commercial production of biopharmaceuticals at least in the short term because they can be established and scaled more rapidly than transgenic plants [53]. We therefore investigated the transient expression of IL6 in tobacco plants and found that the recombinant protein could be produced at levels up to 7% of TSP in *N. benthamiana*, approximately three orders of magnitude higher than the transgenic plants (maximum 0.14% of TSP, Table 4). The relatively poor performance of the transgenic plants is likely to reflect the importance of protein degradation over prolonged timescales, which can be overcome by the rapid and prodigious synthesis that can be achieved using viral replicons followed by harvesting after a few days, before significant degradation takes place. This strategy has been deployed successfully with other unstable proteins, including the hepatitis B virus surface antigen (HbsAg), which accumulated at less than 0.01% TSP in transgenic tobacco leaves but up to 0.26% TSP using the MagnICON system [63], and the LTB-MUC1 protein which achieved yields of 3% TSP by transient expression [64]. Protein stability is the main limitation in transient expression because stable proteins can be produced at extraordinary yields [65], e.g. up to 80% TSP in the case of the highly-resistant marker protein GFP [31,66]. The accumulation of recombinant IL6 can possibly be enhanced by expressing them as fusion with partner proteins that improve stability. This has been achieved by expressing proteins with cholera toxin B subunit (CTB), viral coat proteins, ubiquitin, β -glucuronidase (GUS) and human immunoglobulin [23,67]. The fusion to seed storage proteins like natural zein, synthetic elastin-like protein (ELP) and fungal hydrophobins might be beneficial as well. These induce the formation of protein bodies (PB's) as storage organelles in leave tissue, which are devoid of proteolytic activity [67]. The impact of cellular proteases can also be reduced by coexpressing a protease inhibitor, or the development of specialized plant lines lacking protease activity [68–70].

Although the accumulation of IL6 may be restricted by protein instability, the transgene insert may also act to destabilize the viral RNA with a negative impact on yields [71–73].

The commercial tobacco cultivars we tested produced significantly less IL6 in absolute amounts than *N. benthamiana* despite the greater biomass yields under glasshouse conditions, and similar results were achieved with the control protein GFP. *N. benthamiana* is therefore the preferable host for IL6 production. This difference in performance probably reflects the presence of a functional RDR6 enzyme in the commercial cultivars, whereas the same protein is inactive in *N. benthamiana* thus encouraging virus replication [32]. We also noted that both commercial cultivars inhibited viral spreading and were not amenable to agrospraying, which would limit the value of this alternative approach in the field. There was a significant difference in performance between the two cultivars, confirming that recombinant protein expression

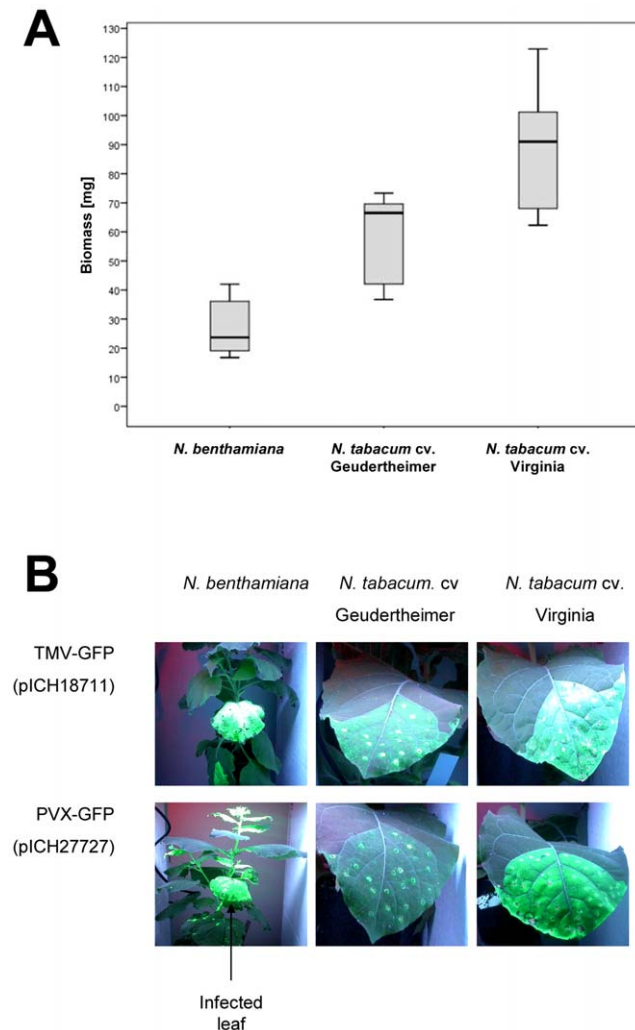


Figure 7. Suitability of different *Nicotiana* cultivars for transient expression. (A) Biomass of 8-week old-plants (*N. benthamiana* and the two commercial tobacco cultivars Geudertheimer and Virginia), used for transient expression assays. (B) Leaves from all three hosts, syringe infiltrated with bacteria carrying GFP vectors pICH18711 and pICH27727, viewed under UV-light at 10 dpi. doi:10.1371/journal.pone.0048938.g007

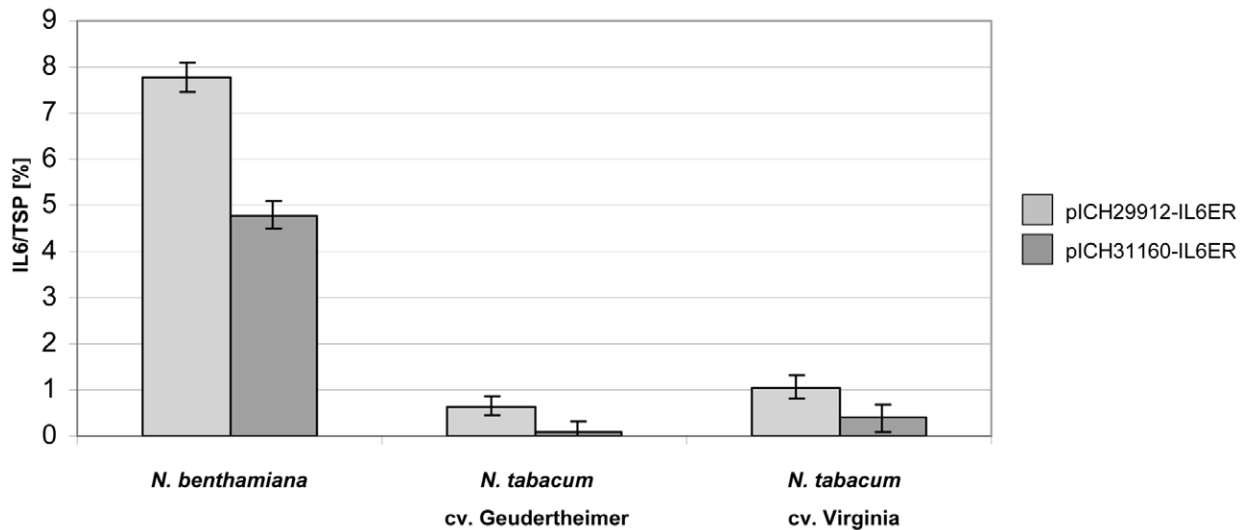


Figure 8. Transient expression of IL6ER in different *Nicotiana* cultivars. *N. benthamiana* and the two commercial tobacco cultivars were infiltrated by syringe with *A. tumefaciens* strain ICF320 carrying vectors pICH29912-IL6ER and pICH31160-IL6ER, respectively. Leaves were sampled at 10 dpi and IL6 levels were determined by ≥ 2 independent ELISA. doi:10.1371/journal.pone.0048938.g008

is affected by both species-dependent and cultivar-dependent factors.

Recombinant IL6 from tobacco is structurally intact and biologically active

Western blots of transgenic leaves and seeds and agroinfiltrated leaves showed that IL6 was present as two dominant bands, matching the profile of native human IL6. The two products of the native protein represent different glycoforms [24]. Plant-derived IL6 might be differentially glycosylated in a similar manner to the native protein, since the potential *N*- and *O*-glycosylation sites of IL6, as described by Orita *et al.* [74], were found to be utilized in plants and humans [75–77]. Though, despite conserved recognition sites, the structure of the glycan moieties differs between humans and plants [75–78]. Nevertheless, additional experiments need to be done in order to prove that the observed bands represent different glycoforms of IL6. Moreover, in case of a glycosylated recombinant protein, it is essential to determine the composition of the putative glycan moiety, since this can affect the

biological activity, stability and pharmacokinetic properties of recombinant proteins [25].

However, absence of glycosylation or glycan differences do not appear to inhibit the biological activity of recombinant IL6 produced in *E. coli* [13,14,16,17] Nausch *et al.*, submitted) or tobacco cytosol [22]. Indeed, our ER-targeted IL6 had an EC₅₀ value of 22 pg/ml, which represents a higher biological activity than the previously-reported cytoplasmic variant with an EC₅₀ value of 50 pg/ml [22].

Plant-specific post-translational modifications may also be targeted by IgE-based allergic responses inducing hypersensitivity reactions [79]. Furthermore, the presence of glycan-specific antibodies in human serum may induce the rapid clearance of glycosylated plant-derived pharmaceutical proteins, which may greatly comprise their *in vivo* efficacy [80]. These aspects have not been investigated for plant-derived recombinant IL6 since the biological activity was determined using an *in vitro* cell assay and the *in vivo* behavior of the protein therefore needs to be investigated prior to application in humans.

Conclusion

We have successfully produced the human cytokine IL6 in a soluble, bioactive conformation in tobacco, providing an attractive alternative to the *E. coli* expression platform, where IL6 is produced either within inclusion bodies [13–17] or as fusion protein [18,19], both of which require labor-intensive downstream processing. The highest yield of IL6 was achieved by transient expression in *N. benthamiana* leaves using the MagnICON system, which can be considered the most appropriate platform for rapid IL6 production in plants on a moderate scale and under containment.

For large scale-production in the field, transgenic seeds accumulating ER-targeted IL6 are preferable because (i) they produce higher yields per fresh weight than leaves, (ii) higher yields than other targeting strategies, (iii) the protein is stable for at least three months, allowing the uncoupling of upstream and downstream production processes, providing flexibility in terms of practical manufacturing issues and economics [27] (iv) the

Table 3. IL6 expression levels (presented as %TSP) using MagnICON vectors in *N. benthamiana* and the two commercial tobacco cultivars Geudertheimer and Virginia.

	<i>N. benthamiana</i>	<i>N. tabacum</i> cv. Geudertheimer	<i>N. tabacum</i> cv. Virginia
TMV-IL6ER	7.78% (±0.30)	0.65% (±0.20)	1.06% (±0.25)
(pICH29912-IL6ER)			
PVX-IL6ER	4.79% (±0.28)	0.09% (±0.22)	0.40% (±0.30)
(pICH31160-IL6ER)			

Standard deviation in brackets.

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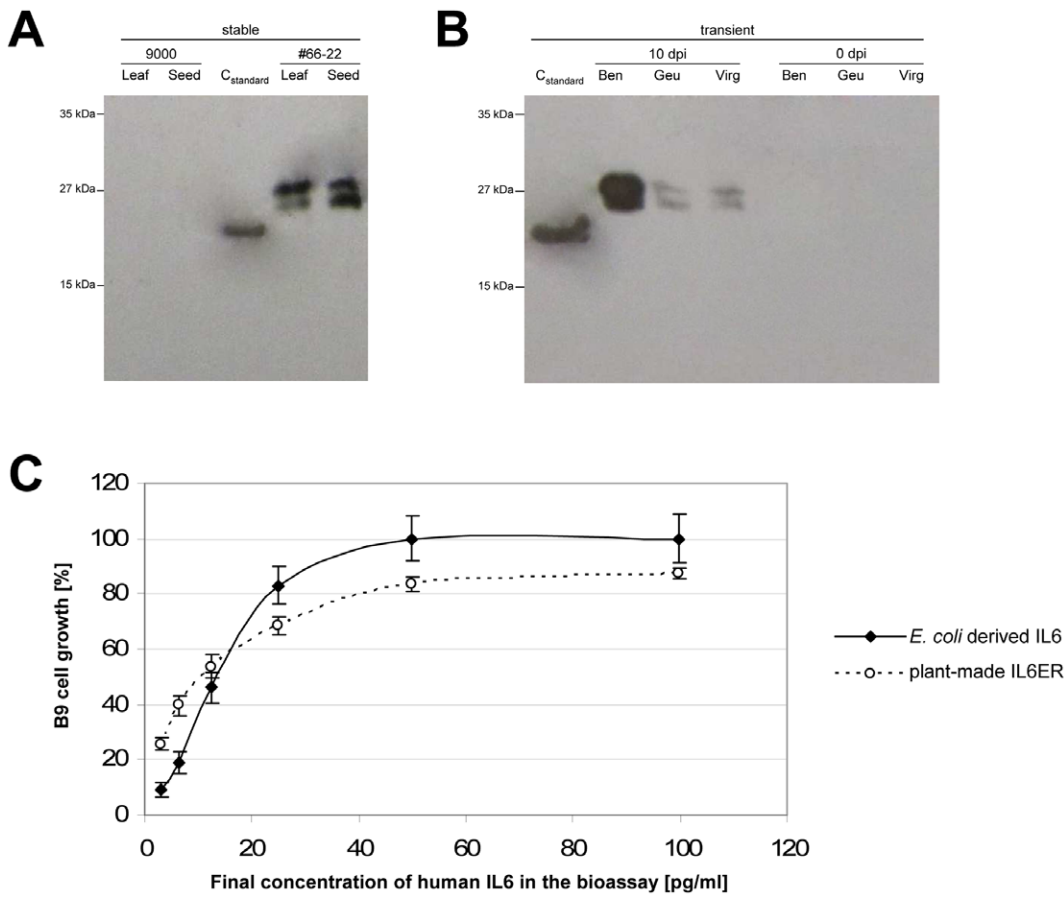


Figure 9. Western blot analysis of recombinant IL6. Analysis of crude leaf extracts from tobacco plants expressing IL6ER either following stable transformation (A) or by transient expression (B). Samples containing 100 µg or 10 µg of total soluble protein (transgenic plants and infiltrated leaves, respectively) were fractionated by 15% SDS-PAGE under reducing conditions, transferred onto a PVDF membrane, probed with mouse anti-human IL6 and visualized using the ECL-system. C_{standard}: recombinant human IL6, expressed in *E. coli*; 9000: control plant transformed with empty binary transformation vector pLH9000 lacking the target gene construct; 66-22: T₁ progeny of a tobacco event transformed with the pLH-IL6ER T-DNA; Ben, Geu, Vir: leaf samples from three different tobacco hosts transfected with the MagniCON vector pICH29912-IL6ER and harvested prior to infiltration (0 dpi) and 10 dpi. (C) Verification of IL6 biological activity using crude leaf extracts from transgenic tobacco compared to a commercial standard IL6 produced in *E. coli*. Cell growth was measured following exposure to different dilutions of the leaf extracts, with the concentration of IL6 determined by ELISA.
doi:10.1371/journal.pone.0048938.g009

Table 4. Comparison of the yields of recombinant IL6 in three different tobacco hosts, achieved by stable or transient expression.

Transformation type	Construct	Cultivar	Plant organ	Expression level [% TSP]
stable	pLH-IL6ER	<i>N. tabacum</i> cv. Geudertheimer	leaf	0.14%
stable	pLH-IL6ER	<i>N. tabacum</i> cv. Geudertheimer	seed	0.12%
transient	pICH29912-IL6ER	<i>N. benthamiana</i>	leaf	7.78%
transient	pICH29912-IL6ER	<i>N. tabacum</i> cv. Geudertheimer	leaf	0.65%
transient	pICH29912-IL6ER	<i>N. tabacum</i> cv. Virginia	leaf	1.06%

doi:10.1371/journal.pone.0048938.t004

cultivation of transgenic plants on an agricultural scale would attract a lower regulatory burden than large-scale spraying with recombinant bacteria [53], and (v) the seeds can be sterilized before processing, making them more attractive for the production of biotherapeutics with respect to GMP compliance [27]. The best performing plants carry a single transgene locus, which allows the straightforward propagation of homozygous transgenic lines.

These aspects of upstream production become even more relevant in relation to the downstream processing, which represent approximately 80% of the overall production costs [28]. The ease and efficiency of product recovery differs drastically between cultivars or organs used for expression [28]. Hence, upon purification the yield might be drastically different compared to

the expression achieved *in planta* with a strong impact on the process economics. Seeds are characterized by a simple metabolic profile and low proteolytic activity, facilitating downstream purification efforts, whereas leaf tissue produces cross-purifying secondary metabolites and recombinant proteins are highly instable upon harvest [81].

Author Contributions

Conceived and designed the experiments: HN HM UM IB JH. Performed the experiments: HN. Analyzed the data: HN HM JH. Contributed reagents/materials/analysis tools: UM IB. Wrote the paper: HN. Performed bioassay: RK.

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