Expression and Subcellular Targeting of Human Complement Factor C5a in *Nicotiana* species

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**Abstract**

We evaluated transgenic tobacco plants as an alternative to *Escherichia coli* for the production of recombinant human complement factor 5a (C5a). C5a has not been expressed in plants before and is highly unstable *in vivo* in its native form, so it was necessary to establish the most suitable subcellular targeting strategy. We used the strong and constitutive CaMV 35S promoter to drive transgene expression and compared three different subcellular compartments. The yields of C5a in the T₀ transgenic plants were low in terms of the proportion of total soluble protein (TSP) when targeted to the apoplast (0.0002% TSP) or endoplasmic reticulum (0.0003% TSP) but was one order of magnitude higher when targeted to the vacuole (0.001% TSP). The yields could be increased by conventional breeding (up to 0.014% TSP in the T₂ generation). C5a accumulated to the same level in seeds and leaves when targeted to the apoplast but was up to 1.7-fold more abundant in the seeds when targeted to the ER or vacuole, although this difference was less striking in the better-performing lines. When yields were calculated as an amount per gram fresh weight of transgenic plant tissue, the vacuole targeting strategy was clearly more efficient in seeds, reaching 35.8 μg C5a per gram of fresh seed weight compared to 10.62 μg C5a per gram fresh weight of leaves. Transient expression of C5aER and C5aVac in *N. benthamiana*, using MagnICON vectors, reached up to 0.2% and 0.7% of TSP, respectively, but was accompanied by cytotoxic effects and induced leaf senescence. Western blot of the plant extracts revealed a band matching the corresponding glycosylated native protein and the bioassay demonstrated that recombinant C5a was biologically active.

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**Introduction**

Autoimmune and inflammatory diseases (AIDs) are characterized by an overactive immune system. They are becoming more prevalent in society and more of a significant challenge to health authorities around the world. Particularly sepsis following bacterial infection often fail standard medical treatments due to the spread of antibiotic resistance [1]. With mortality rates exceeding 70%, sepsis is one of the top 10 causes of death worldwide [1–4]. Therefore, there is a strong demand for novel therapeutic approaches to regulate the immune system. Complement factor 5a (C5a) is a promising candidate for immunomodulatory therapies of sepsis [4–7], since it has been characterised as potent mediator of the innate immune system to infection and ‘key’ mediator of sepsis and septic organ dysfunction [1,4–12]. Though, the development of this strategy is hampered by the lack of an efficient production system for recombinant C5a.

Recombinant pharmaceutical proteins are usually produced by fermentation in *Escherichia coli*, yeast, insect cells or mammalian cells, each with particular advantages and disadvantages often depending on the importance of post-translational modifications for therapeutic efficacy [13]. C5a produced in *E. coli* accumulates as inclusion bodies and therefore requires laborious solubilisation and refolding to achieve the native confirmation [14–16]. Attempts to express soluble C5a in *E. coli* have only been partially successful [17]. The solubilisation of inclusion bodies is undesirable in commercial downstream processing because of the increased process time and costs [18], therefore *E. coli* is not suitable for the commercial production of C5a.

In contrast to microbes, plants can fold and modify complex human proteins and should therefore be able to produce C5a in a soluble and active form [19]. Plants also have the advantage of economy, scalability and increased safety compared to animal cells, since they do not support the replication of human pathogens [20,21]. Among the many plant species used for the production of recombinant proteins, tobacco (*Nicotiana tabacum*) is regarded as a major emerging platform for the production of certain pharmaceutical products, especially subunit vaccines and antibodies [22–24]. Tobacco is a leafy crop that produces up to 100 tons of leaf biomass per hectare and the total soluble protein (TSP) content is higher than in many other plant species [19]. Tobacco is neither a food nor a feed crop, thus reducing the likelihood of transgenic
material contaminating the food or feed chain. Tobacco has also proven to be compatible with the demands of good manufacturing practice (GMP), which is critical for the regulatory approval of plant-derived pharmaceuticals [25]. Proof of concept has been demonstrated for various antibodies, subunit vaccines, hormones and enzymes, including other blood factors which have successfully been produced in tobacco with a yield of up to 0.15% TSP in leaves [19]. Several plant-derived pharmaceutical proteins have successfully completed phase I clinical trials to demonstrate safety, and many plant derived vaccines have also demonstrated efficacy by inducing a significant immune response [23,26].

Our objective was to demonstrate the usage of tobacco to produce recombinant human C5a. We compared different targeting strategies for their impact on protein yields, since it has been demonstrated in many previous studies that targeting proteins to different tissues and subcellular compartments is more successful than letting them accumulate in the cytosol or testing a single tissue [27]. We therefore compared proteins targeted to the apoplast, endoplasmic reticulum (ER) and protein storage vacuoles (PSVs) in leaves and seeds. We selected the commercial tobacco cultivar N. tabacum cv. Geudertheimer as the production host because of its superior biomass yield. In addition, as alternative to stable transformation, we investigated the feasibility of the MagniICON-based transient expression system for the expression of C5a in N. benthamiana.

Materials and Methods

Construction of plant expression vectors

We designed a synthetic C5a coding region based on the 74-amino-acid sequence of the human complement factor C5a, a cleavage product of the precursor protein C5 (accession no. -P01031|678aa|Fig. S2). This was codon optimized for expression in tobacco (http://www.kazusa.or.jp). The coding region was supplemented with a 28-amino-acid N-terminal ER-targeting peptide from the human IL6 gene (accession no. P05231-1) as shown in figure S1. We also added three codons (GCT TCC TCG) after the ATG initiation codon to improve the efficiency of translation [28]. These constructs were synthesized by the DNA Cloning Service (Hamburg, Germany).

We used the binary transformation vector pLH9000 (accession no. AF415478) [29] containing the neomycin phosphotransferase type II gene (nptII) for selection [30] and 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 [31], the tobacco mosaic virus (TMV) Ω-fragment [32] and the CaMV terminator [31] at the SfiI site, and then integrated the above-mentioned synthetic gene constructs at the BamHI/EcoRI sites in the polylinker. The protein expressed using this basic cassette was targeted to the apoplast. For ER and vacuolar targeting, synthetic oligonucleotide sequences were designed based on the SEKDEL ER-retention signal [33], and the vacuole sorting determinant AFVY from phaseolin [34] as shown in figure S3. The synthesized oligonucleotides (Invitrogen) were fused to the 3'-end of the coding region at the MunI/BsrG1 sites. All vectors were verified by DNA sequencing (GATC Biotech AG, Konstanz/Germany).

We used transient expression vectors based on Tobacco mosaic virus (c-TMV/TVCV) provided by Prof. Dr. Yuri Gleba and Dr. Anatoli Giritch (Nomad Bioscience; Halle/ Saale, Germany). These are derivatives of pICH18711, which has been optimized for high yields [35]. The pICH18711 vector is similar to pICH29912 except the green fluorescent protein (GFP) coding region has been inserted into the BsaI cloning site of pICH29912. The coding region of C5aER and C5aVac was incorporated together with flanking BsaI restriction sites into the vector pLC by DNA Cloning Service Hamburg. The coding regions were inserted into the BsaI site of pICH29912 as described [36]. The vectors were verified by sequencing with primer pairs TMV-fw and TMV-rv (Table 1). The transient expression vectors were introduced into Agrobacterium tumefaciens strain ICF 320, a disarmed, auxotrophic derivative (ΔcysKa, ΔcysKb, ΔhldG) of strain C58 [37].

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 several times with sterilized distilled water to remove the detergent and allowed to germinate on 4.4 g/l Linsmaier and Skoog (LS) medium including vitamins (catalog no. L0230.0050; Duchefa, Belgium) supplemented with 30 g/l sucrose and 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 [38] but optimized for the transformation of cultivar Geudertheimer by Tina Hausmann (personal communication). Regenerated shoots were selected on LS medium containing 100 μg/ml kanamycin and 500 μg/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. tabacum (6-9 weeks old) was carried out as described by Giritch et al. [39]. A bacterial smear was inoculated into 5 ml starter culture containing 50 μg/ml rifampicin and 50 μg/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 5.3) 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 μl of extraction buffer (50 mM NaOH, 0.25% SDS). After boiling for 10 min and pelleting in a bench centrifuge, the supernatant was diluted 1:5 in distilled water. PCR was used to detect both the C5a gene and the nptII marker. After an initial denaturation step (95°C for 5 min) we carried out 39 amplification cycles (95°C for 1 min, 56°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 confirmed by PCR against the endogenous actin sequence, which generates different products from genomic DNA and cDNA templates [40]. We used the RevertAid™ H Minus First Strand cDNA Synthesis Kit
same parameters described for DNA amplification, but we used heating to 70°C for 10 min before hybridization. Membranes were washed twice with 0.1% SDS, 1% SSC, 0.1% sodium lauroyl sarcosinate at 42°C for 20 min. Signal detection with the corresponding biotinylated detection antibody at room temperature for 2 h. After another wash, the plates were 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. Following five washes with PBS containing 0.05% Tween-20, the absorbance was measured at 450 nm in a Synergy HT multidetection reader (BioTek, Bad Friedrichshall, Germany).

**Western blot analysis**

Total soluble protein was extracted from leaf and seed samples as described above and 100 µg of protein was resuspended in 1× sample buffer containing 10% glycerol, 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-µm 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 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 C5a antibody (catalog no. MA1-25341, ABR BioReagents, USA) or rabbit polyclonal anti-human C5a antibody (catalog no. 5995-100; BioVision, Milpitas, USA), each at 1:1000 dilution at room temperature overnight and was then probed at room temperature for 2 h with a mouse monoclonal anti-human C5a antibody (catalog no. MA1-25341, ABR BioReagents, USA) or rabbit polyclonal anti-human C5a antibody (catalog no. 5995-100; BioVision, Milpitas, USA), each at 1:10000 dilution at room temperature for 2 h. After three washes, the membrane was probed at room temperature for 2 h with a 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. Carrier-

**Table 1. Primers used for PCR and RT-PCR analysis.**

| Forward-Primer | Reverse Primer | Product length |
|----------------|---------------|----------------|
| NtC5a-fw 5’-CCTGCCTGATTTTCTGCGAC-3’ | NtC5a-rv 5’-ACGACACAGCACCCTGTGAAGG-3 | 166 bp |
| NtC5a-fw 5’-CCTGCCTGATTTTCTGCGAC-3’ | NtC5aER-rv 5’-GCTATCCCTTCAGACAGTC-3’ | 268 bp |
| NtC5a-fw 5’-CCTGCCTGATTTTCTGCGAC-3’ | NtC5aVac-rv 5’-GATCACAGAAGACAGCTG-3’ | 268 bp |
| 2npt-fw 5’-TCCGGCTGTTGTTGAGAG-3’ | 2npt-rv 5’-CTGCGTGGAGGCCTCTGAGTC-3’ | 450 bp |
| Actin-fw 5’-GCAACTGTGATATGAGAA-3’ | Actin-rv 5’-GTCGCTTCTGAACACAGACG-3’ | 850 bp |
| TMV-fw 5’-GATCCGGACGTGAAGTTCTGAGG-3’ | TMV-rv 5’-CCTGACTCTAGCTAGACGGCGCCCTG-3’ | 977 bp/971 bp* |

*pcCH29912-C5aER and pcCH29912-C5aVac.

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(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 [41], 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 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 phosphate buffer (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-strand DIG-labeled DNA probes were prepared by PCR with construct-specific primers (Table 1) using the corresponding binary vectors as the template and the DIG DNA Labeling Kit (Roche Mannheim, Germany). 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)**

Leaf samples (150 mg) were homogenized in liquid nitrogen and resuspended in 250 µl cold protein extraction buffer (250 mM sucrose, 50 mM Tris (pH 7.5), 1 mM EDTA, 2 mM PMSF, 0.1% Triton X-100). For seed samples, 150 mg homogenized seeds were resuspended in 500 µl 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).

Recombinant C5a was quantified using a commercial Human Complement Component C5a DuoSet ELISA (catalog no. DY2037; R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer’s instructions. Briefly, 96-well plates were coated with a mouse anti-human C5a-specific antibody at a final concentration of 1 µg/ml at room temperature overnight. Following five washes with PBS containing 0.05% Tween-20, the plates were incubated with 100 µl diluted leaf extract at room temperature for 2 h. After another wash, the plates were incubated with streptavidin conjugated to horseradish peroxidase at room temperature for 1 h, washed again and incubated with tetramethylbenzidine 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 (BioTek, Bad Friedrichshall, Germany).
free recombinant human C5a produced in *E. coli* (catalog no. 2037-C5-025/CF, B&D Systems, Heidelberg, Germany) was used as a standard.

**Determination of C5a biological activity**

C5a activity was detected using a rat basophilic leukemia (RBL)-cell line transfected with human C5aR which was developed by Ali *et al.* [42]. The cell line was provided by MBM ScienceBridge GmbH (Göttingen, Germany). The bioassay for lysosomal enzyme secretion was conducted as described by Goldstein and Weissmann [43]. The amount of antigenic C5a, applied to the bioassay, was calculated on the basis of the C5a-specific ELISA, which detects the number of antigenic C5a per ml. After incubation with recombinant C5a, the cells are induced to secrete N-acetyl-β-D-glucosaminidase which releases 4-nitrophenolate from the substrate 4-nitrophenyl-N-β-D-glucosamide. The photometric detection of 4-nitrophenolate indicates the concentration of active recombinant protein, which can then be defined as an EC<sub>50</sub> value. Carrier-free recombinant human C5a produced in *E. coli* (catalog no. 2037-C5-025/CF, B&D Systems, Heidelberg, Germany) was used as a standard.

**Statistical methods**

Statistical comparisons were carried out using the *F*-test (ANOVA including the Bonferroni post-hoc test) with *p*≤0.05 (two-sided) considered significant. The variability of different events and siblings was characterized by the relative coefficient of variation (CV, %) \( V_r[\%] = \frac{s}{\sqrt{n}} \times 100 \). The experimental design was calculated with SPSS.
yield of C5a was approximately an order of magnitude lower in the apoplast and ER variants, respectively 1.6 and 2.9 pg/mg TSP. We determined the coefficient of variation (CV) to measure the degree of variability between individual transgenic events (Fig. 2b). This was highest for the C5aVac construct (CV = 12.66%) whereas both C5aApo (CV = 3.86%) and C5aER (CV = 6.38%) showed relatively low variability.

In order to investigate the accumulation of C5a in seeds and subsequent generations of plants, the ten best-performing T0 plants representing each construct were self-pollinated allowing the collection of T1 seeds. Pools of mature seeds weighing 150 mg were harvested from each T0 event, and extracts of total soluble protein were analyzed by ELISA. Using TSP as the reference parameter (Fig. 3), the average yield of C5aApo was 1.8 pg/mg TSP, which is similar to the level detected in T0 leaves. The yield of C5aER was approximately 1.7-fold higher in seeds (4.9 pg/mg TSP compared to 2.9 pg/mg TSP in leaves; Fig. 4a) and that of C5aVac was approximately 1.5-fold higher in seeds (15.5 pg/mg TSP compared to 10.6 pg/mg TSP in leaves; Fig. 3). The difference between leaves and seeds was less striking in the higher-performing plants, e.g. the yield of C5a in the T1 seeds of the best-performing line C5aVac 19 (18.5 pg/mg TSP) was only slightly higher than the yield in leaves (17.4 pg/mg TSP) as shown in Table 2.

Using the fresh tissue weight as the reference parameter, C5a yields were approximately four times higher in seeds than leaves.
regardless of the targeting strategy. Using C5aVac 19 as example, the average yield in leaves was 1.49 mg/g fresh weight, compared to 4.63 mg/g in seeds (Table 2). The CV values for the variability of expression among different lines were similar to those observed in leaves.

Dry T1 seeds were stored at room temperature for three months and the C5a levels were determined again by ELISA, revealing no significant change in abundance compared to freshly-harvested seeds (data not shown).

Accumulation of C5a in the leaves and seeds of subsequent generations of plants

Descendants of the three best-performing T0 transformants (C5aVac 19, 24, 73; see Fig. 3) were self-pollinated to obtain transgenic plants up to the T2 generation, which were selected on kanamycin to ensure the locus remained active. Most of the T1 descendants of C5aVac 19 and C5aVac 73 accumulated more C5a than their parents, but there was no generational increase in line C5aVac 24 (Fig. 4a). The expression levels among T1 siblings were relatively heterogeneous, with CVs of 15.08%, 13.64% and 12.66% for lines C5aVac 19, 24 and 73, respectively. Some T1 plants accumulated less recombinant protein than their parent (e.g. C5aVac 19-15, which produced 10.6 pg/µg TSP) whereas others produced substantially more (e.g. the best-performing plant C5aVac 19-13, which produced 132.8 pg/µg TSP, corresponding to 10.62 mg/g fresh leaf weight (Table 2)). C5aVac 19-13 was self-pollinated and C5a levels were measured in the T2 seeds. The average expression level was 143.2 pg/µg TSP or 35.8 mg/g fresh weight (Table 2) which represents a near eight-fold increase in seeds over one generation.

Only two individual T2 plants (C5aVac 19-13-1 and 19-13-15) performed better that their T1 parents (Fig. 4b). Leaves from the best-performing plant (C5aVac 19-13-1) produced 140.3 pg/µg TSP or 11.22 mg/g fresh leaf weight (Table 2). The expression level among the T2 siblings were less heterogeneous (CV of 7.17) compared to the T1 individuals (CV of 15.08). The yield of C5a in the leaves of line C5aVac 19 increased by an order of magnitude between the T0 and T1 generations but there was only a marginal increase in the T2 generation (18.3→132.8→140.3 pg/µg TSP) was not significant. The accumulation of C5a in the seeds of plant C5aVac 19-13-1 (121.7 pg/µg TSP or 30.4 mg/g fresh seed weight) was slightly lower compared to its T1 parent (143.2 pg/µg TSP). Most T2 descendants of C5aVac 19-13 accumulated less C5a than the T1 parent, although the variability in expression levels was lower (CV = 7.17% for T2 compared to 15.08% for T1).

Transgene insertions in the lines C5aVac 19, 24 and 73

We analyzed the transgene locus structure in T0 plants from lines C5aVac 19, 24 and 73, and in T1 progeny with the highest

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**Table 2.** Expression levels in leaves and seeds of the best-performing line C5aVac 19 and transiently expressed C5aVac, using either TSP or fresh weight as the reference parameter.

| Construct | Stably transformed line C5aVac 19 | Transient expression of C5aVac |
|-----------|-----------------------------------|-------------------------------|
| Generation | T0 | T1 | T2 | T2 |
| Individual | 19 | 19-13 | 19-13-1 | |
| Organ | leaf | seed | leaf | seed | leaf | seed | leaf |
| C5a/TSP [pg/µg] | 17.4 | 18.5 | 132.8 | 143.2 | 140.3 | 121.7 | 6985.8 |
| C5a/fresh weight [mg/g] | 1.49 | 4.63 | 10.62 | 35.80 | 11.22 | 30.43 | 558.86 |

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**Figure 3.** Average protein levels in the leaves and seeds of ten transgenic T0 tobacco plants expressing different C5a variants, using TSP as the reference parameter, based on at least two independent ELISAs.

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Figure 4. Protein expression levels in the T1 generation of three independent transgenic tobacco lines accumulating C5a in the vacuole, as determined by at least two independent ELISAs. (A) Expression level measured in the individual descendants; Lane numbers represent the different T1 individuals and T0 represents the parent. (B) Southern blots of the C5aVac 19 T0 parent and selected T1 and T2 progeny (identified by lane numbers) and corresponding C5a expression levels determined by ELISA. The genomic DNA was digested with HindIII, which cuts once in the expression vector, and the presence of segregating bands indicates of three unlinked loci in the T0 parent. M = DIG-labeled DNA Molecular Weight Marker II (Roche), NIV DNA from wild-type negative control plant.

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and lowest expression levels between the corresponding siblings (Fig. 4a), namely 19-2, -7, -8, -13, -16 and 24-4, -6, -8, -13, -16 and 73-3, -9, -10, -14, -15. We further analysed the T2 individuals 19-13-1, -2, -3, -8, -13, -14, -18 (Fig. 4b). We detected three transgene loci in the T0 plant C5aVac 19, and the two best-performing T1 plants from this line (C5aVac 19-13 and 19-16) retained all three loci whereas the other three lines (C5aVac 19-2, 19-7 and 19-8) retained only two loci (Fig. 4b). In the T2-generation the highest expression was observed in plants with all three but also with only two loci. As expected for lines with high copy number, the segregation analysis of C5aVac 19; 19-2; 19-13-1; 19-13-15 revealed always nearly 100% transgenic offspring. Hence no correlation between the number of transgene loci and the performance of individual plants was detectable. There were also three transgene loci in the T0 plant C5aVac 73 and these were retained in all five T1 plants we tested (data not shown). Given the highly variable expression levels among these T1 plants, the locus structure did not appear to affect the performance of individual plants. There was a single locus in T0 plant C5aVac 24 and in all five T1 individuals we tested (data not shown).

**Transient expression of C5aER and C5aVac in N. benthamiana, using the MagnICON system**

For transient expression, the coding regions of C5aER and C5aVac were introduced into the MagnICON vector pICH29912 (Fig. 1b), which was kindly provided by Prof. Dr. Yuri Gleba and Dr. Anatoli Giritch (Nomad Bioscience; Halle/Saale, Germany). We selected these two C5a variants, since in stable transformed plants, the C5aApo and C5aER led to similarly low levels of recombinant protein while C5aVac revealed significantly higher yield. The tobacco cultivar Geudertheimer, which was used for stable transformation, has been recorded to be not amenable for transient expression assays and known to promote high level expression of stable transformation, has been recorded to be not amenable for stable transformation, has been recorded to be not amenable for transient expression assays and known to promote high level expression of viral replicons [45, 46]. However, overexpression of both C5a variants was accompanied by cytotoxic effects and induced leaf senescence whereas no severe phenotype was observed in plants infected with the GFP control vector pICH18711 (Fig. 5a). Moreover, we observed a rapid decline in the yield of the target protein in the dying leaves (Fig. 3b). Nevertheless, the highest accumulation level measured at 6 dpi at the beginning leaf senescence reached up to 0.7% of TSP for C5aVac and 0.2% of TSP for C5aER (Table 2). The difference in the accumulation level between transiently expressed C5aER and C5aVac was similar to the one observed in stable transformed tobacco.

**Molecular and functional characterization of recombinant plant-derived C5a**

Western blot analysis of TSP extracts from leaf and seeds samples of transgenic C5aVac plants (Fig. 6a) and transgenic N. benthamiana plants (Fig. 6b) showed a distinct band that migrates at 12 kDa, which is larger than the recombinant product from E. coli (9 kDa) suggesting that the plant-derived recombinant protein might be glycosylated. Crude leaf extracts from the T2 individual C5aVac 19-13-1 and the near isogenic variant as control were used for C5a bioassays based on a RBL-cell line, transfected with an C5a-receptor. The cell line releases an enzyme upon binding of biological active C5a which can be measured by substrate conversion at 405 nm. The concentration of C5a which induced the half-maximal enzyme release was determined as EC50 value. Interestingly, extracts of the near isogenic variant (niv) that served as control converted the substrate as proven by the incubation of mere leaf samples with the substrate (data not shown). Nevertheless, spiked with the C5a standard the NIV induced an additional enzyme release in a dose-dependent manner similar to the commercial C5a standard (Fig. 6c). Hence the basic level of conversion observed in the NIV without additional enzyme was subtracted from the values measured for the transgenic variants. The EC50 value of both samples was calculated at approximately 200 ng/ml. The different concentrations of C5a in the assays were adjusted based on the values measured by C5a-ELISA, in the transgenic leaf and seed extracts. Leaf extracts of C5aVac 19-13-1 induced enzyme release in a manner comparable to the C5a standard but the EC50 value of 279.48 ng/ml was higher (Fig. 6c). Samples of transgenic plants and seeds and from leaves derived from the transient expression in N. benthamiana, resulted in an equal EC50-value (data not shown). Incubating the transgenic leaf samples with the anti-C5a-antibody, used for the Western Blots, prior to the bioassay, reduced the response to the level of the NIV extract (Fig. 6c).

**Discussion**

We have demonstrated for the first time that the human complement factor C5a can be produced in a soluble and active form in stable transformed tobacco, although the yields were low compared to other recombinant proteins expressed using the CaMV 35S promoter [19, 47]. This may reflect the fact that C5a is an unstable protein that is rapidly degraded in the plasma following its release from the precursor C5 [48, 49]. The degradation of recombinant proteins in plants is a relatively common occurrence that limits accumulation in plants, and it is possible that unstable human proteins are also susceptible to endogenous plant proteases [50, 51].

Targeting recombinant proteins to different tissues and subcellular storage compartments can significantly influence the protein stability and yield [27, 52]. In the case of C5a, the vacuole appears to be the most suitable storage compartment, since the highest yields were achieved in leaves and seeds when appending the recombinant protein with the PSV-specific targeting peptide AFVY (Fig. 2a, b). This result was unexpected in leaves because PSVs are underrepresented in vegetative tissues, whereas lytic vacuoles are prevalent that do not support protein accumulation [53]. However, it has been demonstrated that the expression of seed storage proteins in leaves can induce the formation of storage organelles in vegetative tissue [54].

Many heterologous proteins accumulate to higher levels when targeted for retention in the ER because this compartment contains abundant molecular chaperones to fold proteins correctly and maintain their solubility but there are few proteases [50]. This contrasts with the apoplast, where abundant proteases often cause significant degradation [50, 55]. Interestingly, we did not find a significant difference between these compartments and C5a
Figure 6. Western Blot analysis of leaf and seeds samples of transgenic Geudertheimer plants (100 μg TSP); (A) Samples from stable transformed plants; E: 5 ng of recombinant C5a produced in *E. coli* Control: leaf and seed samples from wild-type tobacco. C5aVac: leaf and seed samples of the T2 individual C5aVac 19-13-15, expressing the vacuolar variant of C5a; (B) Samples from *N. benthamiana* at 6 dpi after Agroinfiltration; control: *N. benthamiana* transfected with the empty vector pICH29912; C5aVac: *N. benthamiana* transfected with pICH29912-C5aVac; E: 5 ng of recombinant C5a produced in *E. coli*; (C) Verification of C5a biological activity using crude leaf extracts from transgenic tobacco and the near isogenic variant (wildtype) compared to a commercial standard C5a produced in *E. coli*. Enzyme release was measured by substrate conversion following exposure to different dilutions of the lead extracts, with the concentration of C5a determined by ELISA. doi:10.1371/journal.pone.0053023.g006
accumulation was an order of magnitude lower than in the vacuole. This suggests that C5a is specifically degraded in the ER because it is sensitive to the limited number of highly-specific proteases contained therein. Recently, this phenomenon has also been recorded for other proteins recombinant proteins [56–50]. The higher yields of vacuolar C5a indicate that the recombinant protein may be partially protected by virtue of its vacuolar destination, perhaps due to a shorter residence time in the ER. The vacuole therefore appears to be an appropriate target for recombinant proteins expressed in plants if they are susceptible to proteases in the secretory pathway.

Seeds accumulated 1.4–1.7 more C5a than leaves when the protein was targeted to the ER or the vacuole, probably reflecting the lower proteolytic activity [50] and the higher protein concentration in seeds 25% compared to 10% in tobacco leaves [59,60]. There was no difference between seeds and leaves when the protein was targeted to the apoplast, probably because this does not function as a storage compartment in either tissue. Interestingly, the only previous publication known to us that compares the accumulation of proteins in tobacco leaves and seeds using the CaMV 35S promoter, reported yields of up to 5% of TSP in leaves and only 0.4% in seeds [61]. Since the transgene encoded protein is a relatively stable antibody, this difference might reflect the promoter activity, which is known to be less active in seeds compared to leaves [62]. Assuming, that C5a is more stable in seeds compared to leaves this might balance the lower activity of the promoter.

We noted a sharp increase in protein accumulation in both leaves and seeds between the T₀ and T₁ generations, which might reflect homozygosity of the transgene loci for the individuals 19-13 and 19-16 since the same integration loci are present. Phenomena like these have already been demonstrated [63]. However, the increase seems to be too pronounced to reflect only the doubling of the copy number. In addition, since all offspring of a selfed homozygous line should be homozygous, the T₂ generation should show similar expression levels, which is not the case. Hence other factors might influence the increase in transgene expression, which has also been reported by others [64,65].

The protein levels in the seeds were stable for more than three months providing a strong advantage over leaves, where proteins degrade soon after harvest and must be extracted promptly [50]. Tobacco seeds also lack many of the secondary metabolites that degrade soon after harvest and must be extracted promptly [50]. Months providing a strong advantage over leaves, where proteins

The C5a isolated from tobacco had a significantly higher molecular weight than the E. coli standard (Fig. 6). This might reflect the addition of N-linked glycans at the Asn64 residue, which is also glycosylated in the native human protein [72]. In fact, the potential N-glycosylation sites were found to be conserved between plants and animals [73–75]. The assumption that plant derived C5a is glycosylated is also supported by the fact that, the EC50 value of plant-derived C5aVac of 279.46 ng C5a equivalents/ml was different compared to that of E. coli-derived counterpart with 192.03 ng C5a equivalents/ml [17]. Using the plant extract spiked with E. coli derived C5a we could prove that this difference is not due to endogenous metabolites or enzymes present in the crude extract. Nevertheless, additional experiments need to be done in order to prove that plant-derived C5a is glycosylated.

Supporting Information

**Figure S1** Sequence of the IL6 signal peptide including three triplets downstream the initiator codon ATG (underlined) that increase the efficiency of recognition. (DOC)

**Figure S2** Sequence of the mature C5a gene product, derived from the precursor C5. (DOC)

**Figure S3** Compartment specific C-terminal variants of C5a codon-optimized for tobacco. (DOC)

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Author Contributions

Conceived and designed the experiments: HN HM IB. Performed the experiments: HN. Analyzed the data: HN HM IB JH. Contributed reagents/materials/analysis tools: IB UM. Wrote the paper: HN HM IB. Performed the C5a-bioassay: RK.
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